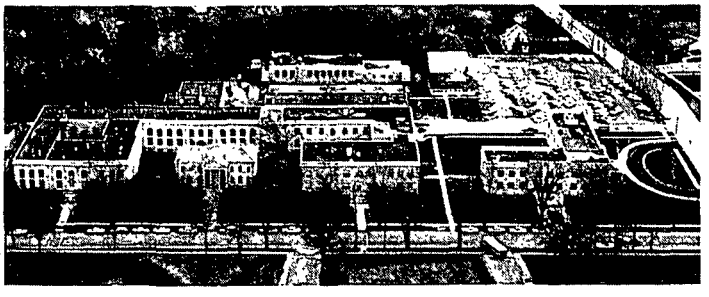

THE INSTITUTE OF PAPER CHEMISTRY, APPLETON, WISCONSIN

P A C

FOREST GENETICS

RESEARCH COMMITTEE MEETING

HANDOUTS

March 25-26, 1985

AGENDA

Project Advisory Committee
Forest Genetics
March 25-26, 1985

The Institute of Paper Chemistry
Continuing Education Center
Appleton, Wisconsin 54912

MONDAY, MARCH 25, 1985

9:00 a.m.	A. Introduction and Overview	Einspahr
	1. Review of PAC recommendations	
	2. Review of project plans and accomplishments for past six months	
	3. Recent publications	
	4. Committee business	
10:20 a.m.	Coffee Break	
10:30	B. Model Systems Research	
	1. Introduction	Einspahr
10:50	2. Polyamines, enzymes and embryogenesis	Feirer
11:30	3. Polyamine inhibitors, seed and embryo development in pine	Feirer
12:00	4. Coffee - Competent vs. Incompetent Sources	Feirer
12:20	Lunch	
1:00	5. Use of natural pine cone extracts	Johnson
1:20	6. Tannin content, PAL levels and phenolics studies with pine and wild carrot	Johnson
2:00	7. Tracer Studies on Protein vs. Phenolic Metabolism	Johnson

2:45	Coffee Break	
3:00	8. Special Topic - <u>In vitro</u> isolation and propagation of mammatoxin-resistant aspen	Wann
	C. Objective I Research "Generating and Maintaining Quality Cell Suspensions"	
3:45	1. Initiation of gymnosperm cell lines using immature embryos	Einspahr
4:00	2. Effect of synthetic auxins on cell line growth and initiation	Noland
4:20	3. Ethylene in wild carrot and loblolly pine	Noland
4:50	4. Effect of light and density centrifugation on polyamine metabolism in loblolly pine suspensions	Feirer
5:20	Cocktails and Dinner	
	D. Objective II - Embryogenesis Research	
6:30	1. Studies on pine launch factors	Johnson
7:00	2. Unmonitored launch attempts	Johnson
7:20	E. Research Plans	Einspahr
	1. Model Systems	
	2. Objective I Research	
	3. Objective II Research	
	4. Expansion Plans and Staff Time Evaluation	
	F. Related Research	
8:20	1. Cooperative research	Einspahr
8:30	2. Student research	Einspahr

8:40	3. Aspen and larch genetics research	Einspahr
9:00	Adjourn	

TUESDAY, MARCH 26, 1985

7:00 a.m.	Breakfast	
8:00	Agenda	Einspahr
8:10	PAC Deliberations	Lazar
11:30	Adjourn	
11:30	Lunch (CEC Dining Room)	

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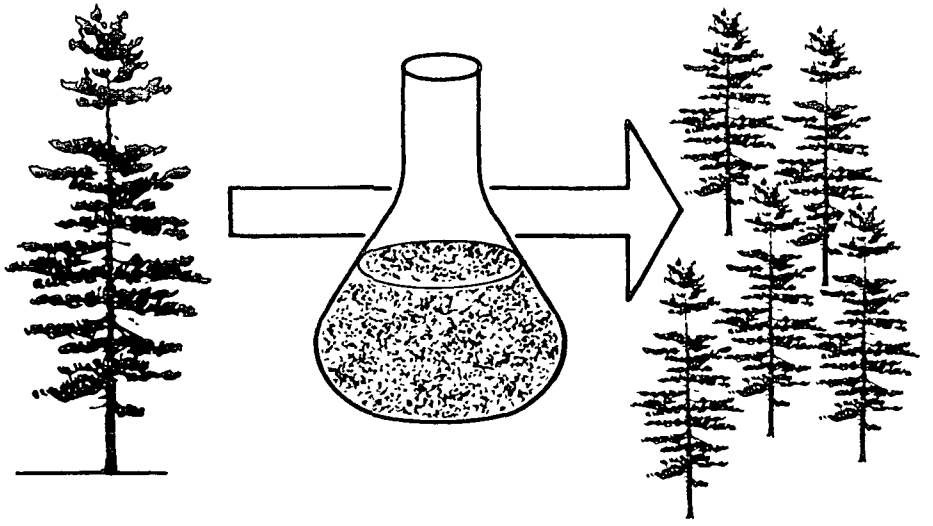
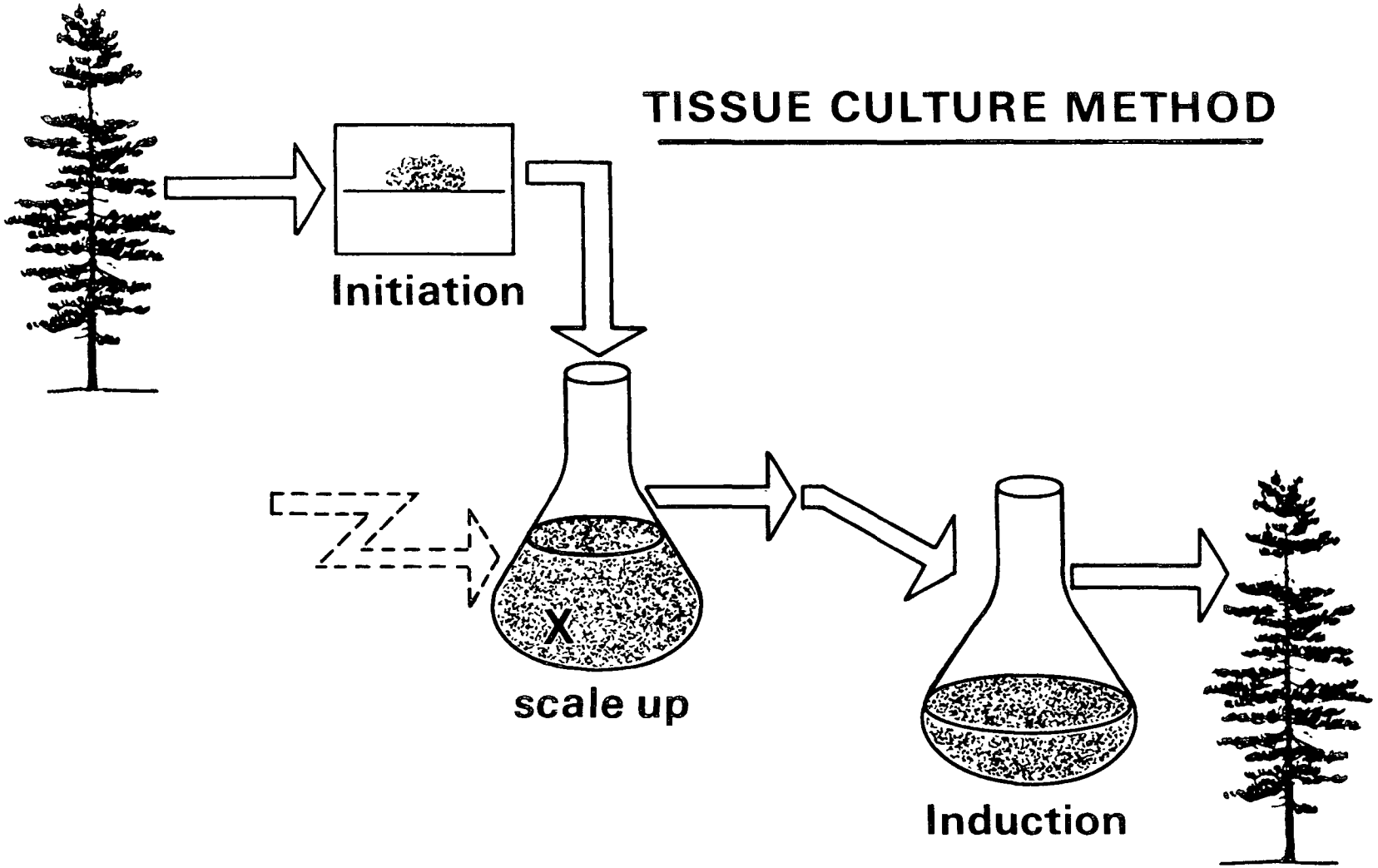
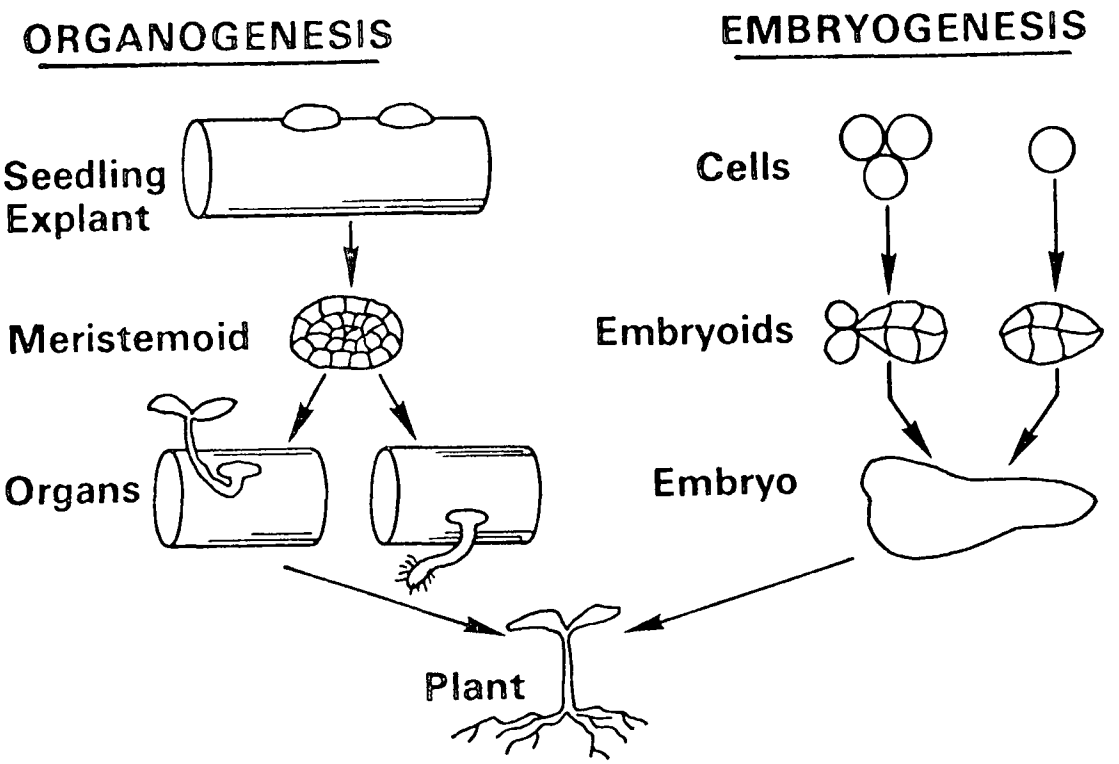
MASS PRODUCTION OF CONIFERS

Loblolly Pine and Douglas Fir




TISSUE CULTURE METHOD
Initiation
X
scale up
Induction

MORPHOGENESIS



SUMMARY - SOMATIC EMBRYOGENESIS

- Defined - production of plantlets from single cells or small groups of cells
- Advantages
  1. Mass production method
  2. Allow efficient use of genetic engineering techniques

PAC RECOMMENDATIONS - OCTOBER 1984

PAC Administrative Recommendations - 10/4/84

- Provide PAC with follow up letter reviewing PAC recommendations
- Review PAC recommendations -- beginning each meeting
- Confine team presentations to salient issues -- using slides to answer critical questions
- Continue "Question and Answer" concept in presenting results

PAC Administrative Recommendations - 10/4/84

- Provide an analysis of staff time
- Provide information on more specific goals for each generalized objective
- Update plans for each meeting emphasizing items that have priority

PAC Administrative Recommendations - 10/4/84

- Provide, in the mailing prior to each meeting, a prioritized list of plans showing accomplishments and future status
- Start meetings with plans and accomplishments and end with listing of future plans
- Provide PAC with brief status report on IPC efforts in conventional forest genetics

PAC Recommendations on Technical Issues - 10/4/84

- Address the issue of the appropriateness of specific model systems
- Because it is clear that loblolly pine is not mimicking wild carrot somatic embryogenesis -- hope to see fewer data on wild carrot
- Discussions on biochemical markers were not totally convincing and the committee felt data could have a number of interpretations

PAC Recommendations on Technical Issues - 10/4/84

- The Committee is not clear to what extent the loblolly pine and wild carrot systems parallel and diverge from each other
- There is concern why certain biochemical work has been started and then set aside
- The committee suggests we use more unusual techniques to illustrate important points

COMMENTS FROM FRED HAAS LETTER

- The committee believes in many instances wild carrot can no longer serve as an appropriate model system -- team should broaden its horizons
- The committee felt some of the differences Russ Feirer has found between wild carrot and loblolly pine further confirmed wild carrot was not an appropriate model species
- The committee felt we should re-examine our results on redox status (ascorbic and dehydro-ascorbic acid) and energy status (ATP and energy charge) before doing additional work in this area

PROJECT 3223 RESEARCH PLANS

1984/85 MODEL SYSTEMS RESEARCH PLANS FEIRER

A. Polyamine Research -- Generate data on the importance of polyamines in natural pine and carrot embryogenesis, wild carrot somatic embryogenesis and in cultured pine cells. Attempt to control metabolism in cultured pine cells.

Accomplishments

1. Confirmed the importance of spermidine in embryogenesis and organogenesis in several plant species.

2. Compared polyamine metabolism in vitro and in intact plants.

3. Initiated studies evaluating relationship between polyamine and ethylene biosynthesis.

4. Demonstrated that light significantly affects polyamine metabolism in cultured cells.

5. Determined that our cell suspensions could be fractionated by centrifugation, and that the cells in the high density heavy fraction had improved appearance and selected biochemical parameters.

Johnson & Noland

B. Phenolics Research -- Characterize and quantify phenolic levels during wild carrot somatic embryogenesis, in loblolly pine cell suspensions and manipulate phenolic levels in wild carrot and loblolly pine.

Accomplishments

1. Pine cells were found to have more persistant and higher levels of PAL enzyme activity than wild carrot cells. PAL is a key enzyme associated with high levels of tannins.

2. Low levels of several phenolic compounds added to the media did not reduce growth of loblolly pine and wild carrot nor embryogenesis in wild carrot. Results with ferulic acid indicate oxidation is probably involved when toxity is observed.

3. A HPLC procedure for separation and quantification of several phenolics has been achieved.

Johnson

C. Growth Regulators (Endogenous) -- Quantify levels of IAA, and other endogenous growth regulators during natural pine embryogenesis, wild carrot somatic embryogenesis, and in loblolly pine cell suspensions.

Accomplishments

1. Personnel limitations have resulted in minimal activity in this research area. Student research on the IAA/indole pathway has opened the possibility of fruitful research in this area.

Johnson

D. Growth Regulators (Synthetic) -- Evaluate alternative synthetic growth regulator to replace 2,4-D in loblolly pine cell suspensions.

Accomplishments

1. Fifteen growth regulators were evaluated for use as alternatives to 2,4-D and a few were promising enough to warrant further testing.

2. Related student research resulted in evidence that the accumulation of 2,4-D by cell lines, as the result of repeated subculturing, may result in cell line incompetence.

Conkey

E. Mitotic Index and Cell Counting-- Determine the usefulness of mitotic index (proportion of dividing cells) as a histological maker for cell line quality. Develop a procedure for determining cell numbers that will allow placing biochemical data on a per cell basis.

Accomplishments

1. Cell counting technique has been perfected.

2. A mitotic index measurement technique has been perfected.

3. Preliminary counting and mitotic index information has been obtained on loblolly pine.

Johnson

F. Energy Research -- Pine cells in suspension may fail to generate and maintain sufficient energy levels for biosynthesis needs. Plans were to determine levels of ATP and energy charge in pine cell suspensions and compare with energy levels in wild carrot and natural pine embryogenesis.

Accomplishments

1. Energy levels (ATP and energy charge) were monitored during wild carrot somatic embryogenesis.

2. Similar measurements were made during natural pine embryogenesis and during attempts to produce somatic embryos using loblolly pine and incompetent wild carrot cell lines.

3. Data suggest pine cells have adequate energy levels but are not using the energy properly.

G. Redox Research -- Pine cells in suspension appear to improperly regulate their internal redox status. Plans were to determine levels of ascorbic and dehydroascorbic acid as a measure of redox status and compare with wild carrot and natural pine embryogenesis.

Accomplishments

1. Measurements of ascorbic acid, dehydroascorbic acid and glutathione were made on wild carrot to obtain an estimate redox status of this system.

2. Similar measurements were made during natural pine embryogenesis and for pine cell suspension during attempts at producing embryos.

3. Data available at this time suggests that the pine cells are too oxidizing during a time period critical for embryo development.

PLANS - OBJECTIVE I RESEARCH

- Generate new cell lines from immature embryos, protoplasts and microsporophyll tissue
- Determine the influence of nitrogen sources, polyamines and growth regulators on cell line quality
- Determine the influence of natural conifer extracts on cell line quality
- Examine the importance of light on cell line quality

Accomplishments - Objective I

1. Several hundred new cell lines were generated from immature loblolly pine embryos, some with improved quality.

2. Polyamine additions to loblolly pine failed to improve cell line quality.

3. Cell lines were initiated using "non-2,4-D" growth regulators.

4. Natural conifer extracts failed to improve cell line quality of loblolly pine.

5. New cell lines have been produced that grow at low 2,4-D levels and low inoculation densities.

6. Several synthetic auxins were tested that have promise and may be less disruptive to subsequent development than 2,4-D.

PLANS - OBJECTIVE II RESEARCH

- Run monitored launch experiments with established cell lines - to correct apparent deficiencies and reduce inhibitors

- Conduct occasional unmonitored launch experiments using promising new ideas

- Conduct monitored launch experiments using promising new cell lines

- Determine the usefulness of natural extracts, new growth regulators, and stress as ways of triggering embryogenesis

Accomplishments - Objective II

1. In preliminary cell suspension studies on N-benzyladenine (BA), uptake was found to be rapid and linear with time.

2. Unmonitored launch attempts to induce somatic embryogenesis were initiated using the 12 best loblolly pine cell lines from 1983 and using six induction media. No organized structures were produced.

3. Unmonitored launches utilizing more than 2000 explants from 1984 immature embryo initiations were established and are being monitored for possible structures.

4. Additional related studies on growth regulator removal and inoculation densities were conducted.

STATUS OF RECENT PUBLICATIONS

1. Monroe, S. H.; Johnson, M. A. A membrane-bound-o-methyltransferase from Douglas-fir needle callus and its role in the nature of endogenous phenolics. Phytochemistry 23(8):1541-3(1984).

2. Feirer, R.; Mignon, G.; Litvay, J. Arginine decarboxylase and polyamines required for embryogenesis in wild carrot. Science 223:1433-5(1984).

3. Feirer, R.; Mignon, G.; Wann, S. Effect of spermidine synthesis inhibitors on in vitro plant development. Plant Physiol. 75:103(1984) Supplement (Poster).

4. Einspahr, D. W. Tissue culture in forestry, current status. Proceedings Second Regional Technical Conference, Soc. of Am. Foresters, Charlotte, NC, Jan. 26-27, 1984:8-16

5. Einspahr, D. W.; Litvay, J. D.; Johnson, M. A.; Feirer, R. P. Challenges of somatic embryogenesis in conifer tissue culture. Proceedings, International Symposium of Recent Advances in Forest Biology. Traverse City, Michigan, June 10-13, 1984:75-81.

6. Litvay, J. D. The Institute of Paper Chemistry approach to propagation of forest trees using somatic embryogenesis. Proceedings, TAPPI R&D Division Conference, Oct. 1-3, 1984:17-19.

7. Litvay, J. D.; Johnson, M. A.; Verma, D. C.; Einspahr, D. W. A new tissue culture medium based on the analysis of conifer ovules. (Being revised for Plant Cell Reports.)

8. Wann, S. R.; Einspahr, D. W. Reliable plant formation from seedling explants of Populus tremuloides (Michx.). (Submitted to Silvae Genetica.).

9. Wann, S. R.; Einspahr, D. W. In vitro isolation and propagation of mammatoxin-resistant aspen. (Submitted to Forest Science).

10. Einspahr, D. W.; Wann, S. R. Use of tissue culture techniques in a hardwood tree improvement program. (Accepted for presentation at the 18th Southern Tree Improvement Conference, May, 1985.)

11. Feirer, R. P.; Wann, S. R.; Einspahr, D. W. The effects of spermidine synthesis inhibitors on in vitro plant development. (Submitted to J. Plant Growth Regulation - invited paper.)

MODEL SYSTEMS USE

The basic premise is that we believe there are a large number of developmental similarities between plant species.

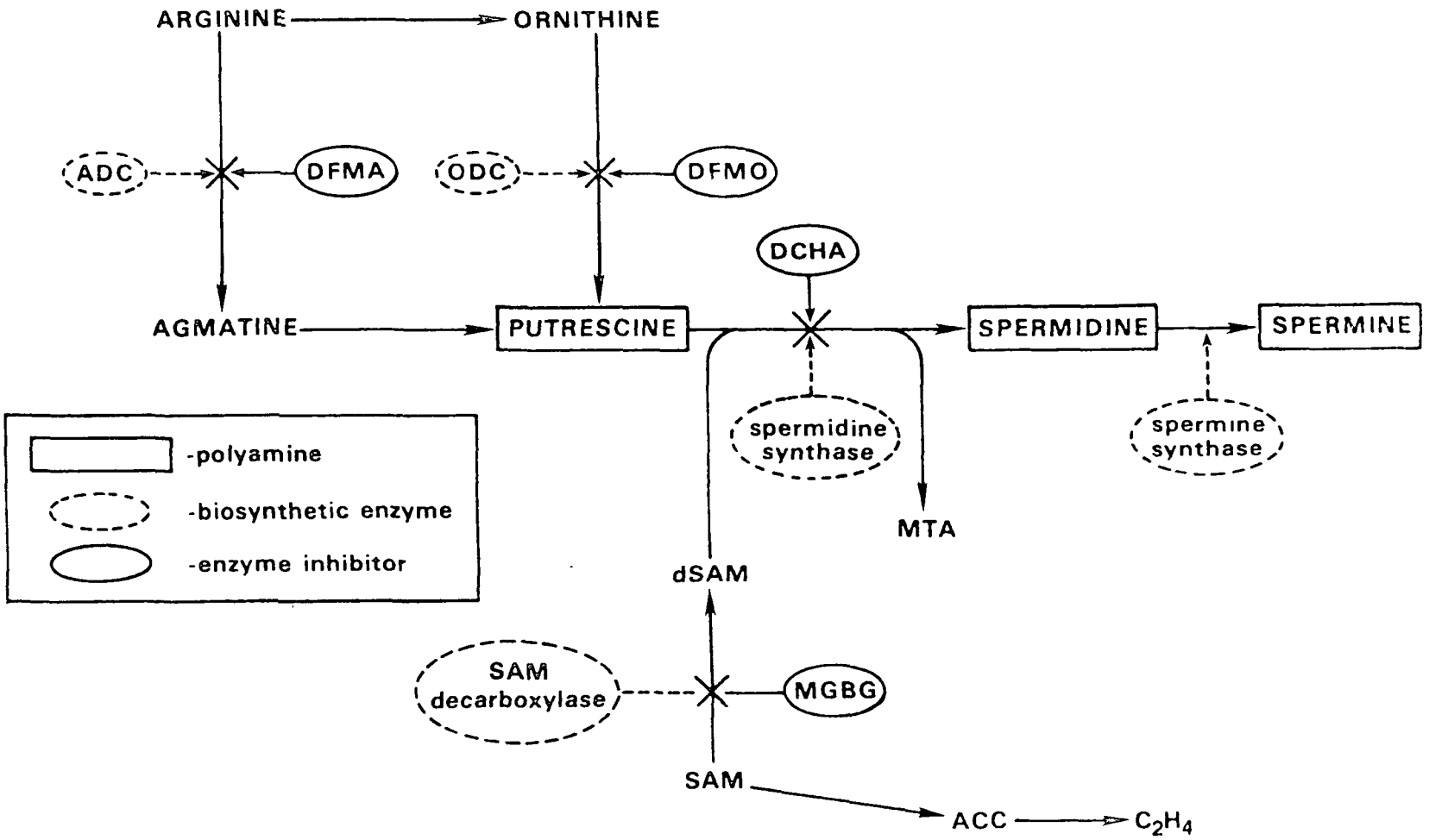
MODEL SPECIES

- Wild Carrot - competent and incompetent lines
- Natural pine embryo development
- Coffee somatic embryogenesis
- Loblolly pine organogenesis
- Literature information on citrus, corn, aspen, soybeans, etc.

SOME IMPORTANT INFORMATION OBTAINED THROUGH THE USE OF MODEL SYSTEMS

- New medium developed for conifers based upon analysis of natural model.
- Importance of cell clump size recognized.
- Importance of inoculation density recognized.
- Small cytoplasm-rich character of embryogenic cells known.
- Morphology to be expected of developing somatic embryos known.
- pH and osmolarity characteristics of embryogenic cell cultures known.
- Possible free amino acid deficiencies of conifer cells appreciated.
- Importance of polyamines in embryogenesis demonstrated.
- Light influences polyamine metabolism.
- ADC enzyme important in carrot somatic embryogenesis. ADC/ODC also appear important in pine embryogenesis.
- PAL enzyme activity and associated tannin production lower in model cells than in pine cells.
- Early overproduction of ethylene occurring in pine cells.
- No apparent energy deficiency in pine cells unless it relates to ATP utilization.
- Internal redox environment of launched pine cells may become too oxidizing too soon.

POLYAMINE METABOLISM IN PLANTS AND INHIBITORS OF BIOSYNTHETIC ENZYMES



Can polyamine metabolism be controlled or manipulated in cultured plant cells?

by:

1. Selection/fractionation
2. Light
3. Growth regulators, etc.

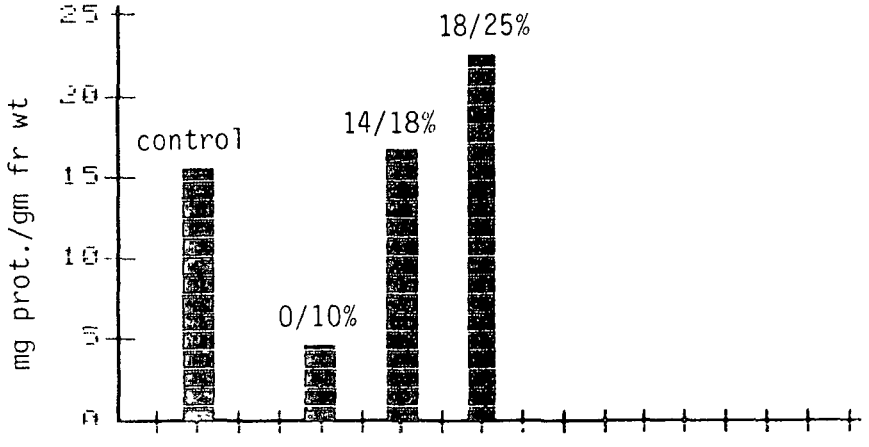
Fractionation of plant cells on basis of density.

1. Possible on Ficoll gradients?
2. Populations exhibit differences in biochemical parameters?
3. Fractions differ in embryo production?

PROTEIN CONTENT IN FRACTIONATED WILD CARROT CELLS

Fraction	mg protein/gm fr wt cells
Control, unfractionated	15.5
0/10% interface	4.5
14/18% interface	16.6
18/25% interface	22.4

Protein content in fractionated wild carrot cells[1]



[1]cell separated on Ficoll gradients of indicated concentrations

POLYAMINE LEVELS IN FRACTIONATED WILD CARROT CELLS[1,3]

Fraction	n[2]	Putrescine	Spermidine	Spermine
Control	3	888[b]	760[b]	64
0/10% interface	1	317	253	N.D.
14/18% interface	3	781[b]	226[b]	52
18/25% interface	2	1918[a]	557[a]	104

[1]nmoles/gm fr wt; cells fractionated in Ficoll gradients.

[2]Limited replication due to amount of cells available.

[3]Values with different superscripts are significantly different ($p<.05$) as compared by Duncan's New Multiple Range Test.

ENZYMES IN FRACTIONATED WILD CARROT CELLS[4]

Fraction	Enzme Activity[1]	
	ADC[2]	ODC[3]
Control, screened >63μ, <279μ	4.3 ± .2[a]	.5 ± .1
0/10% interface	5.7 ± .2[b]	5.8 ± .4[a]
14/18% interface	3.4 ± .1[c]	0.6 ± 0
18/25% interface	5.7 ± .2[b]	0.8 ± .1

[1]nmoles $^{14}CO_2$ released per gm fr wt·hr.

[2]ADC levels followed by different superscripts are significantly different ($p<.05$).

[3]ODC level followed by superscript[a] is significantly different from all other means ($p<.05$).

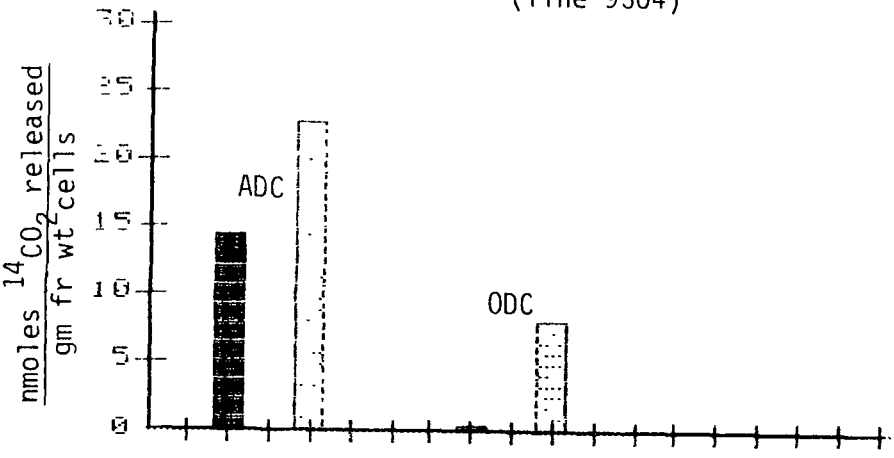
[4]Suspension cells grown in 0.1 mg/L 2,4-D in darkness for 14 days.

Density centrifugation appears to increase:

1. Visual quality (subjective)
2. Protein content
3. Polyamine levels
4. Selected enzymes
5. Embryogenesis (?)

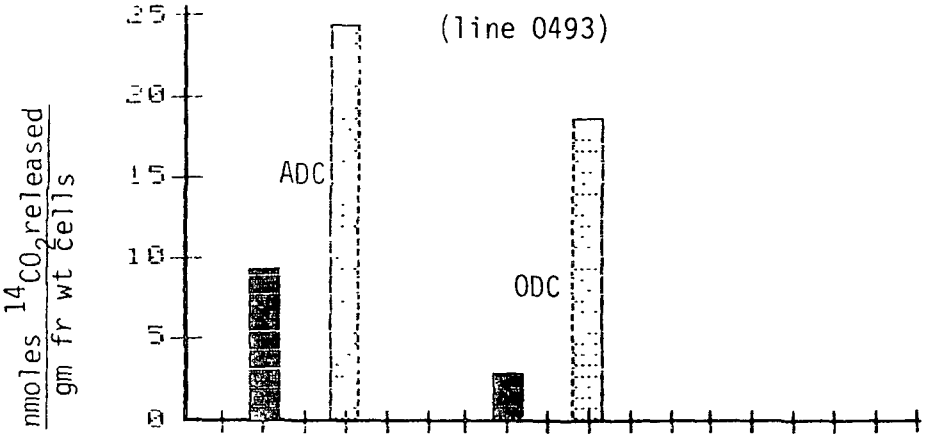
Does light effect polyamine metabolism in cultured plant cells?

Effect of light on enzymes in suspension cultured carrot[1]
(line 9304)



[1]cells grown in MS medium containing 2,4-D (1 mg/L) and kinetin (0.2 mg/L) for 6 days. Open bars represent light grown, dark bars represent dark grown cells.

Effect of light on enzymes in suspension cultured carrot[1]
(line 0493)



[1]cells grown in MS medium containing 2,4-D (1mg/L) and kinetin (0.2 mg/L) for 6 days. Dark bars represent dark grown, open bars represent light grown cells.

EFFECT OF LIGHT ON ENZYMES IN SUSPENSION CULTURED CARROT CELLS[1]

Tissue		Enzyme Activity[2]	
		ADC	ODC
0493	dark	9.3 ± 2.4	2.9 ± 1.0
	light	24.5 ± 2.5	18.6 ± 2.5
intact plant		11.3 ± 1.3	6.9 ± 0.3
9304	dark	14.5 ± 5.7	0.3 ± .1
	light	22.7 ± 4.6	8.1 ± 3.6
intact plant		3.7 ± 0.3	1.4 ± 0.1

[1]Cells grown in MS medium containing 2,4-D (1.0 mg/L) and kinetin (0.02 mg/L) for 6 days.

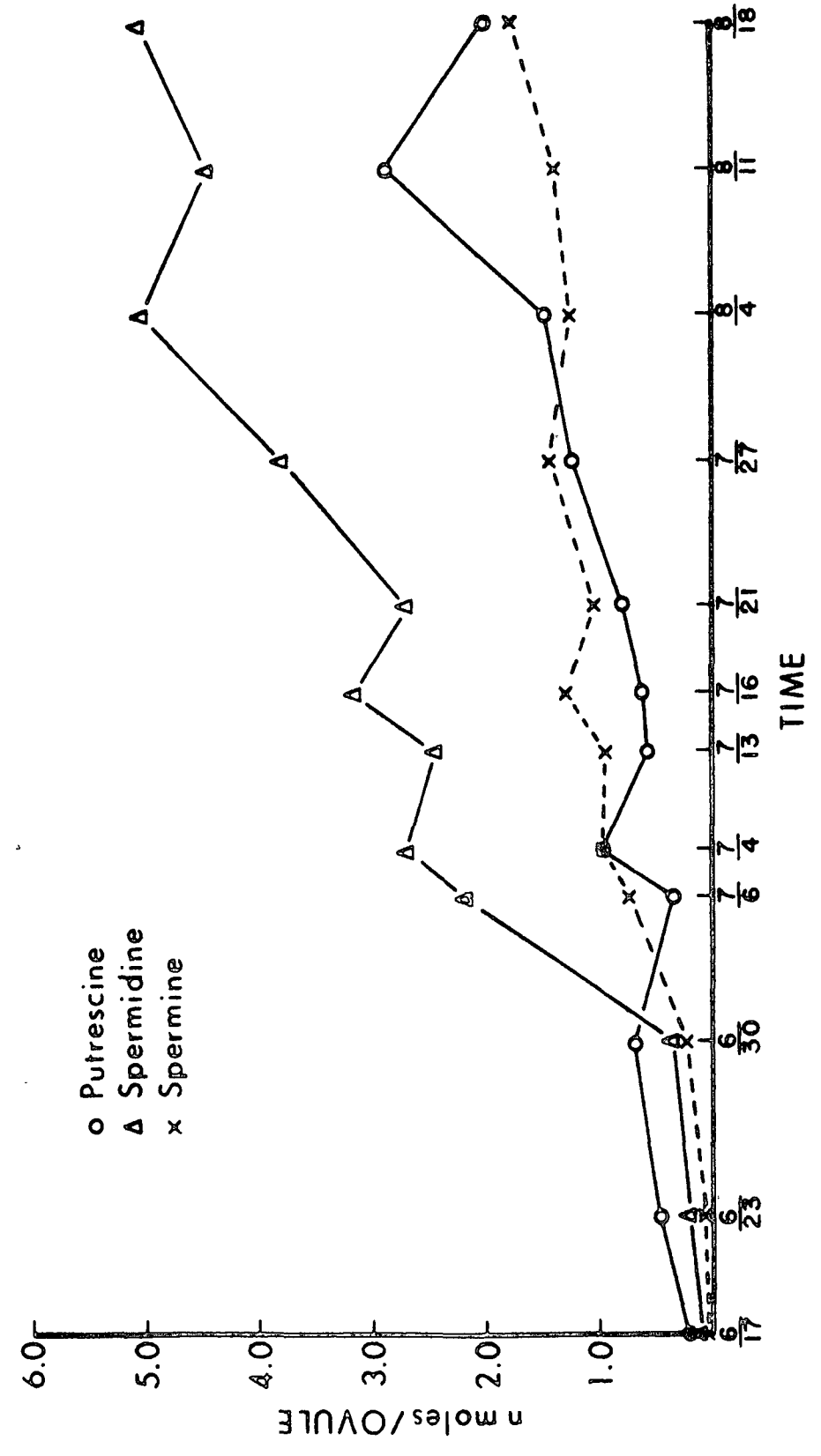
[2]nmoles $^{14}CO_2$ released per gm fr wt·hr.

Does light effect polyamine metabolism in cells?

Light appears to significantly influence polyamine metabolism in (our) plant cells.

Elevated levels of polyamines are essential to wild carrot somatic embryogenesis and are associated with seed development in pine cones. Do polyamines have a major role in seed/embryo development in vivo?

1. Can experimental compounds be delivered?
2. Are polyamines and enzymes affected?
3. Is seed/embryo development affected by polyamine manipulations?



Polyamine levels in developing white pine seeds.

EFFECT OF INHIBITORS OF POLYAMINE BIOSYNTHESIS ON RED PINE SEED DEVELOPMENT

Treatment	Number Cones Treated	Number Seeds/Cone	Number Embryos/Cone
Control-no tube	6	38.3 ± 8.8	33.0 ± 8.3
Control - H_2O	5	42.8 ± 9.8	36.4 ± 8.4
DFMA	5	31.0 ± 3.3[a,b]	26.6 ± 2.7[a,b]
DFMA + putrescine	3	47.0 ± 5.6[b]	40.0 ± 3.5[b]
DFMO	5	32.8 ± 11.8[a]	23.2 ± 16[a]
DFMO + putrescine	3	30.3 ± 2.9[a]	25.0 ± 3.6 [a]
MGBG	4	27.5 ± 1.3[a]	25.3 ± 1.3[a]
MGBG + spermidine	3	35.7 ± 3.1[a]	31.7 ± 5.0

[a]Significantly different from H_2O control, Duncan's New Mutliple Range Test ($p < 0.05$).
[b]Significantly different, ibid ($p < 0.05$).

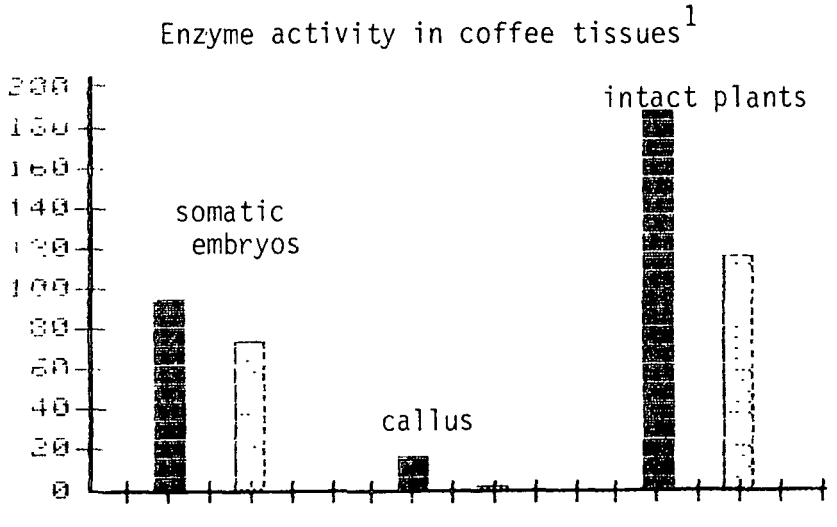
Coffee As A Model System

1. Are polyamines/enzymes higher in somatic embryos than callus?
2. Can ability of culture to form embryos be predicted by levels of polyamines/enzymes in donor plant?
3. What enzymes are responsible for polyamine biosynthesis in coffee?
4. Control of coffee polyamines, ...?

ENZYME ACTIVITY IN COFFEE TISSUES (SUMMARY)

Tissue	Enzyme Activity[1]	
	ADC	ODC
Embryos	94.9 ± 6.3	73.2 ± 6.8
Callus	16.6 ± 7.1	2.5 ± .7
Trees	187.6 ± 45.6	116.1 ± 32.7

[1]nmoles $^{14}CO_2$ released per gm fr wt·hr.



[1]nmoles $^{14}CO_2$ released/ gm fr wt. Dark bars = ADC
Open bars ≙ ODC

ENZYME ACTIVITY IN COFFEE TISSUES

	Enzyme Activity[1]	
Tissue	^{14}C - Arg	^{14}C - Orn
C24 embryos	100.1 ± 3.6	74.4 ± 9.0
C20 embryos	89.7 ± 2.3	72.3 ± 5.6
C19 callus	21.1 ± 4.9	2.3 ± 1.0
C22 callus	12.1 ± 6.4	2.7 ± .3
C24 tree	147.5 ± 5.8	82.2 ± 15.2
C20 tree	220.5 ± 5.2	131.0 ± 27.6
C19 tree	145.5 ± 32.3	96.3 ± 0.7
C22 tree	236.8 ± 5.1	154.6 ± 2.7

[1]nmoles $^{14}CO_2$ released per gm fr wt·hr.

ENZYME ACTIVITY IN COFFEE TISSUES

	Enzyme Activity[1]				
Tissue	^{14}C - Arg	Arg + DFMA	Arg + DFMO	^{14}C - Orn	Orn + DFMO
C24 embryos	100.1 ± 3.6	61.7 ± 8.8	46.1 ± 1.9	74.4 ± 9.0	38.6 ± 2.3
C20 embryos	89.7 ± 2.3	64.1 ± .6	39.5 ± 4.3	72.3 ± 5.6	41.7 ± 2.7
C19 callus	21.1 ± 4.9	2.8 ± .6		2.3 ± 1.0	
C22 callus	12.1 ± 6.4	3.3 ± 1.7		2.7 ± .3	
C24 tree	147.5 ± 5.8	146.2 ± 19.8	122.5 ± 3.3	82.2 ± 15.2	
C20 tree	220.5 ± 5.2	201.9 ± 42.9	165.0 ± 20.6	131.0 ± 27.6	
C19 tree	145.5 ± 32.3	152.4 ± 17.2	157.2 ± 36.2	96.3 ± 0.7	60.2 ± 13.4
C22 tree	236.8 ± 5.1	244.7 ± 7.4	191.9 ± 28.9	154.6 ± 2.7	

[1]nmoles $^{14}CO_2$ released per gm fr wt·hr.

INFORMATION GAINED FROM THE USE OF MODEL SYSTEMS (SUMMARY)

- Cell quality can be improved by fractionation on basis of density
  - Heavy cells exhibit improved appearance, higher protein, polyamine and enzyme levels
- Polyamine metabolism is significantly affected by light
- Polyamines and enzymes leading to their biosynthesis appear to have a major role in natural red pine embryo development
- Preliminary studies with coffee have again suggested that polyamines are involved with plant development (high enzyme levels in somatic embryos)

EXTRACTS OF IMMATURE PINE SEEDS

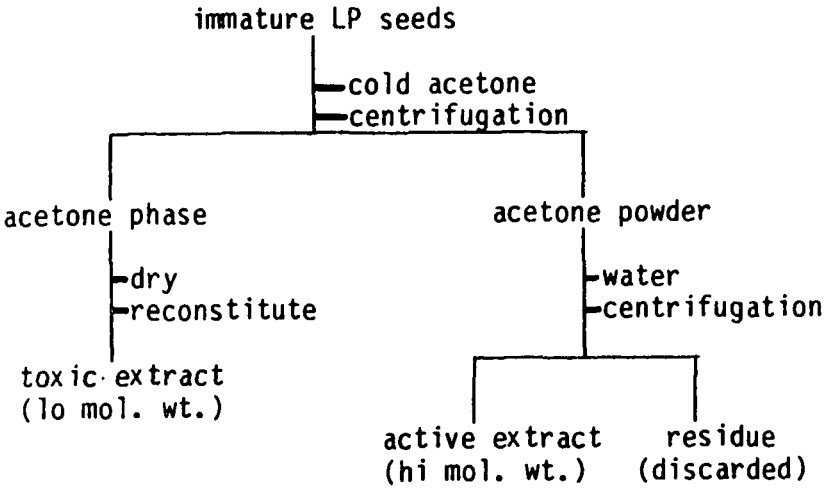
Q. Where do we stand on the use of extracts of immature pine seeds to promote somatic embryogenesis?

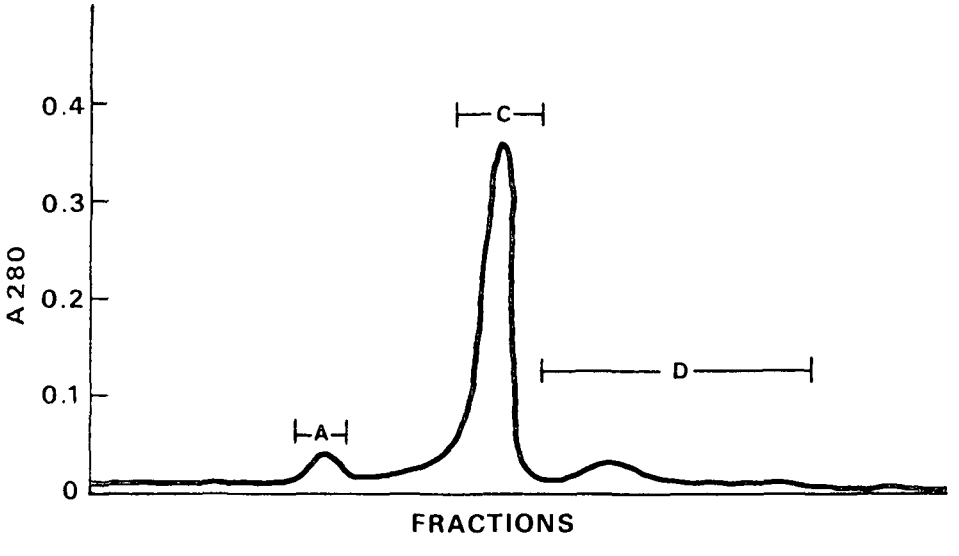
A. Active extract subjected to gel permeation chromatography (a mild fractionation procedure) yielded inactive fractions. The basis for this unexpected result is under investigation. Some testing of pine suspension cell spent media fractions generated in a Ph.D. thesis is also ongoing.

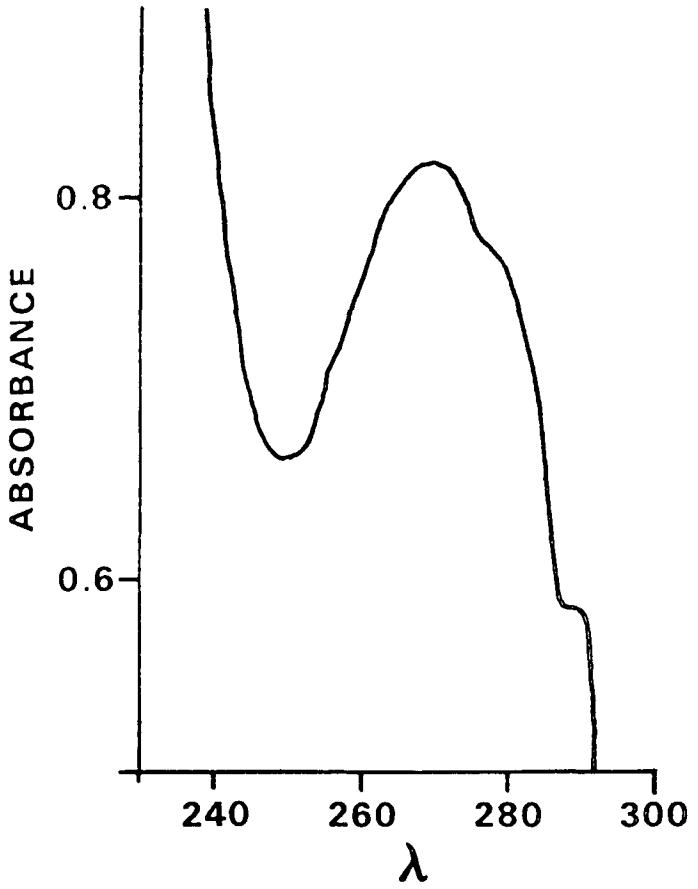
Effects of LP seed extracts on wild carrot somatic embryogenesis.

Sample	Embryos, numbers/tube[a]	Fresh Wt., mg/tube[a]
Buffer control	31.4 ± 11.2^{b}	23.8 ± 6.1^{c}
1983 Extract in buffer	127.0 ± 30.5^{a}	119.2 ± 21.3^{a}
1984 Extract in buffer	54.8 ± 15.2^{b}	52.1 ± 9.4^{bc}
1984 Extract in water	121.0 ± 23.8^{a}	120.2 ± 22.1^{a}
Water control	44.5 ± 8.5^{b}	35.3 ± 5.2^{bc}

[a] $\bar{x} \pm$ SD (n = 5 except water control where n = 4). ANOVA and Duncan's Multiple Range Test were used to compare the sample means for embryo counts and fresh weights; values with common superscripts are not significantly different at the 95% confidence level.


immature LP seeds
cold acetone
centrifugation
acetone phase
acetone powder
dry
reconstitute
water
centrifugation
toxic extract
(lo mol. wt.)
active extract
(hi mol. wt.)
residue
(discarded)


0.4
0.3
0.2
0.1
0
A 280
A
C
D
FRACTIONS



Effects of BioGel P-10 fractions and deproteinized extract on wild carrot somatic embryogenesis.

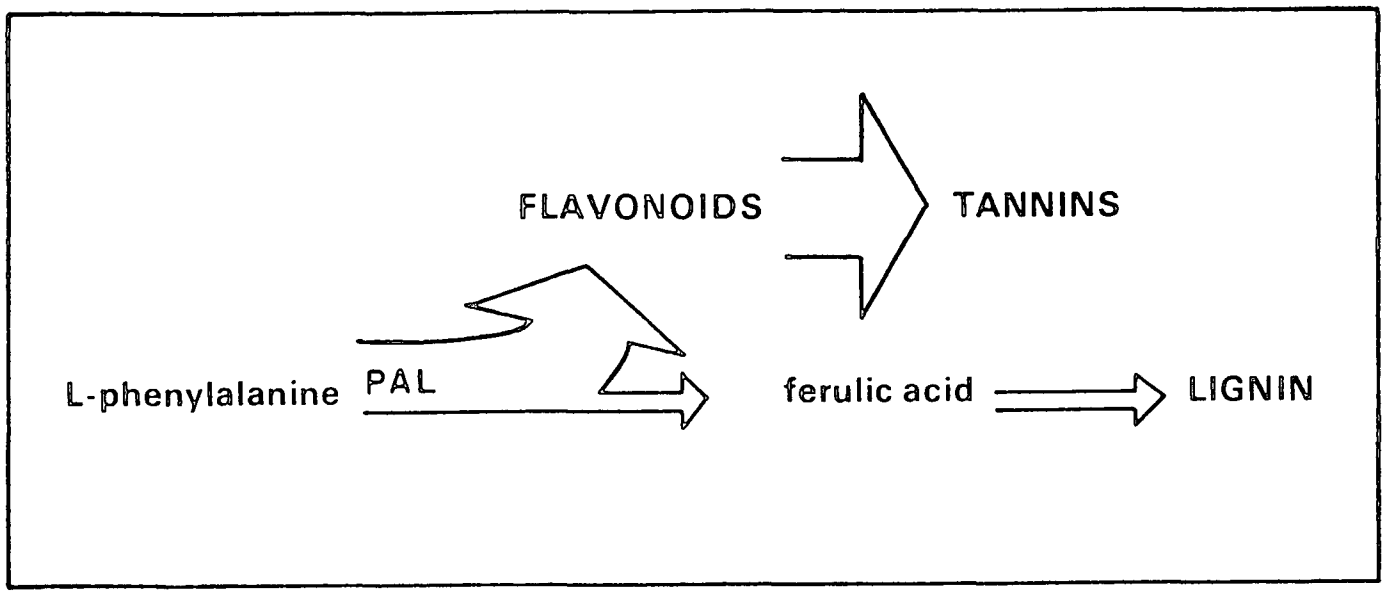
Treatment	Embryo Yield (14 days)	
	Embryo Counts[a]	Fresh Weight, mg[a]
Control	82 ± 8^{cd}	124 ± 35^{abc}
Extract	124 ± 12^{a}	157 ± 19^{a}
Deproteinized extract	109 ± 10^{b}	141 ± 30^{ab}
Fraction A, 0.01 mg/mL	66 ± 6^{efg}	124 ± 22^{abc}
Fraction A, 0.1 mg/mL	77 ± 11^{cde}	103 ± 40^{cd}
Fraction C, 0.01 mg/mL	55 ± 11^{fg}	111 ± 21^{bcd}
Fraction C, 0.1 mg/mL	86 ± 8^{c}	127 ± 13^{abc}
Fraction D, 0.01 mg/mL	71 ± 11^{def}	114 ± 14^{bcd}
Fraction D, 0.1 mg/mL	51 ± 6^{g}	83 ± 18^{d}

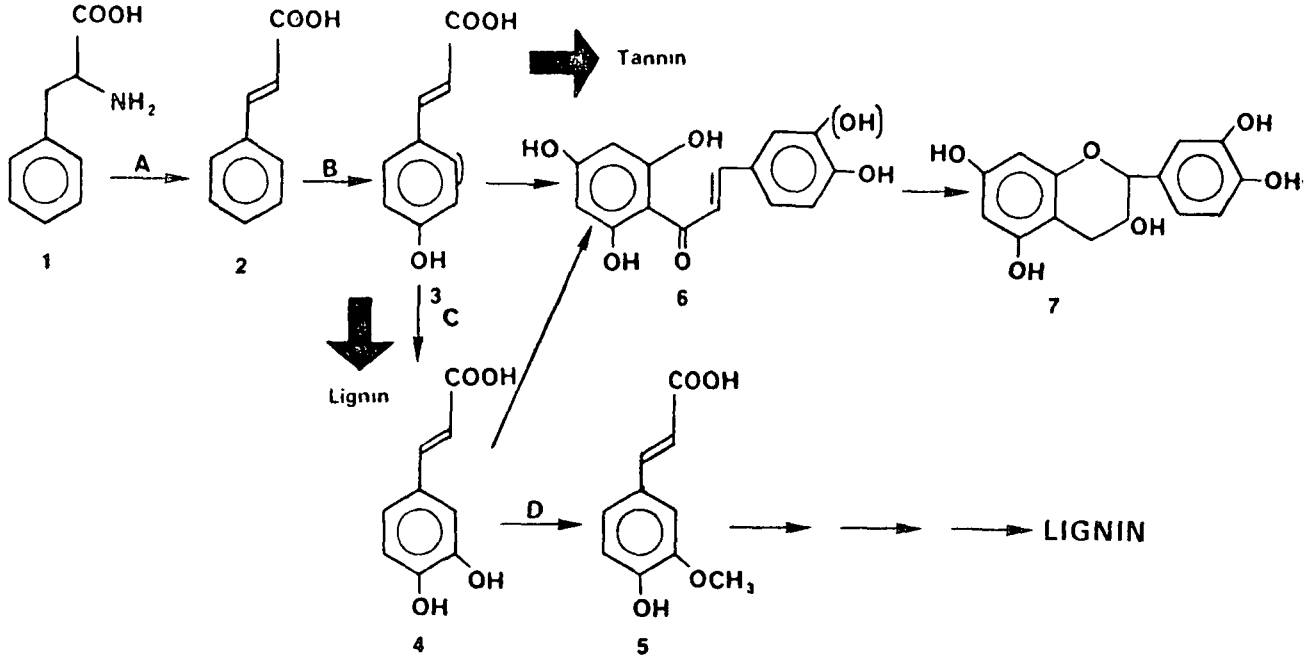
[a] $\bar{x}$ ± SD (n = 5). ANOVA and Duncan's Multiple Range Test were used to compare the treatment means for embryo counts and fresh weights; values with common superscripts are not significantly different at the 95% confidence level.

PHENOLIC STUDIES

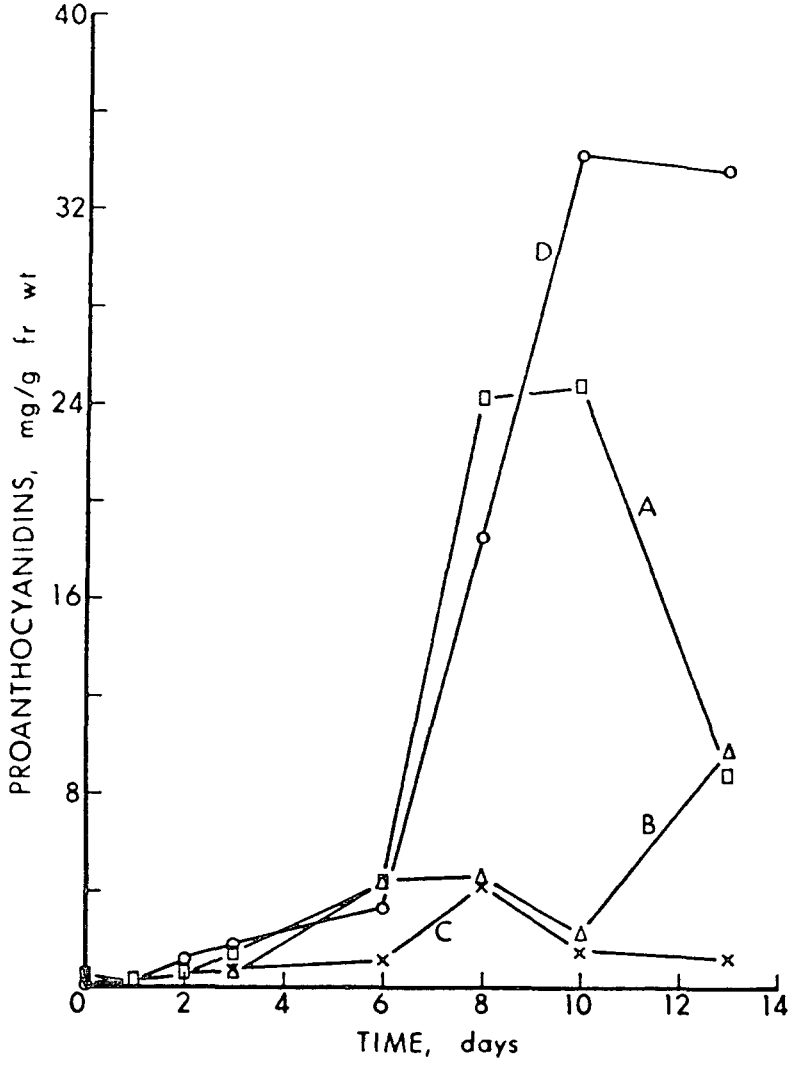
Q. How do the wild carrot model cells compare with cultured conifer cells in terms of phenolic metabolism and response to added phenolics?

A. Although wild carrot cells make little if any tannin during a culture period, they do respond to transfer to fresh medium with an increase in PAL activity. The PAL activity of wild carrot is weaker and less persistent than in pine cultures and yields a different net result. Testing the response of carrot and pine suspension cells to various added phenolics shows that, at low concentrations, many low molecular weight phenolics are quite compatible with proliferative growth and embryogenesis in wild carrot and with proliferative growth in pine. Phenolic problems in pine launch experiments would appear to be dependent upon a failure of pine cells to keep these compounds reduced under launch conditions.

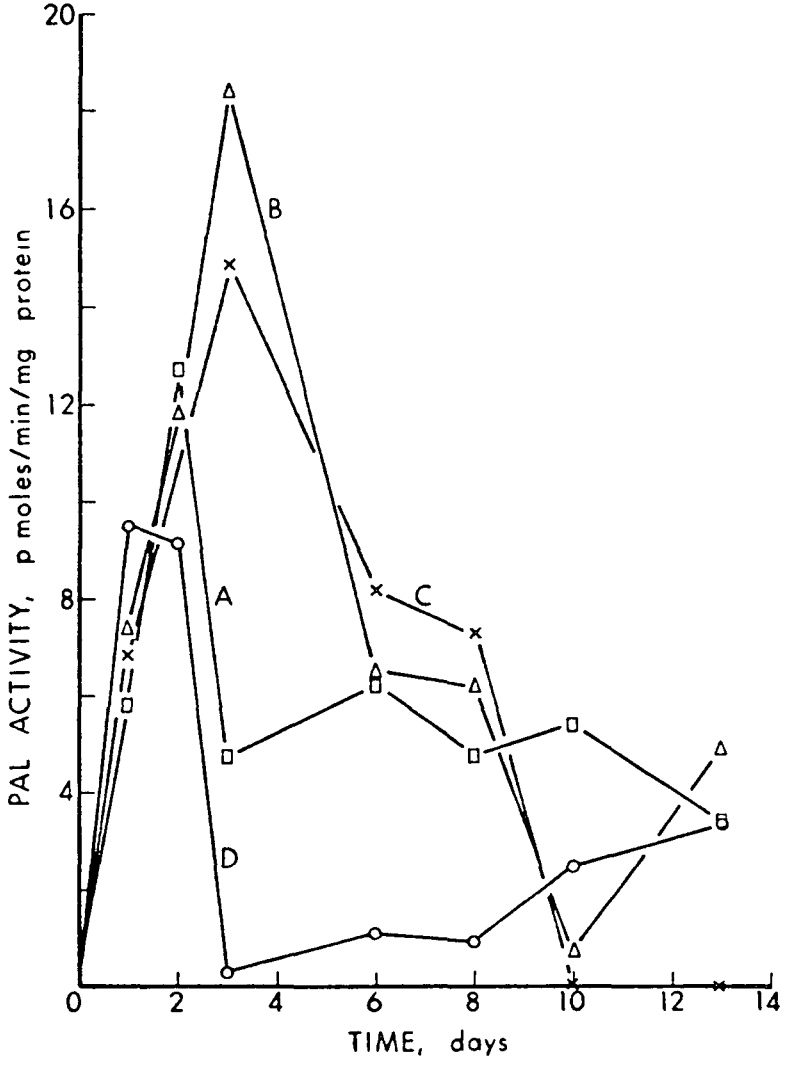




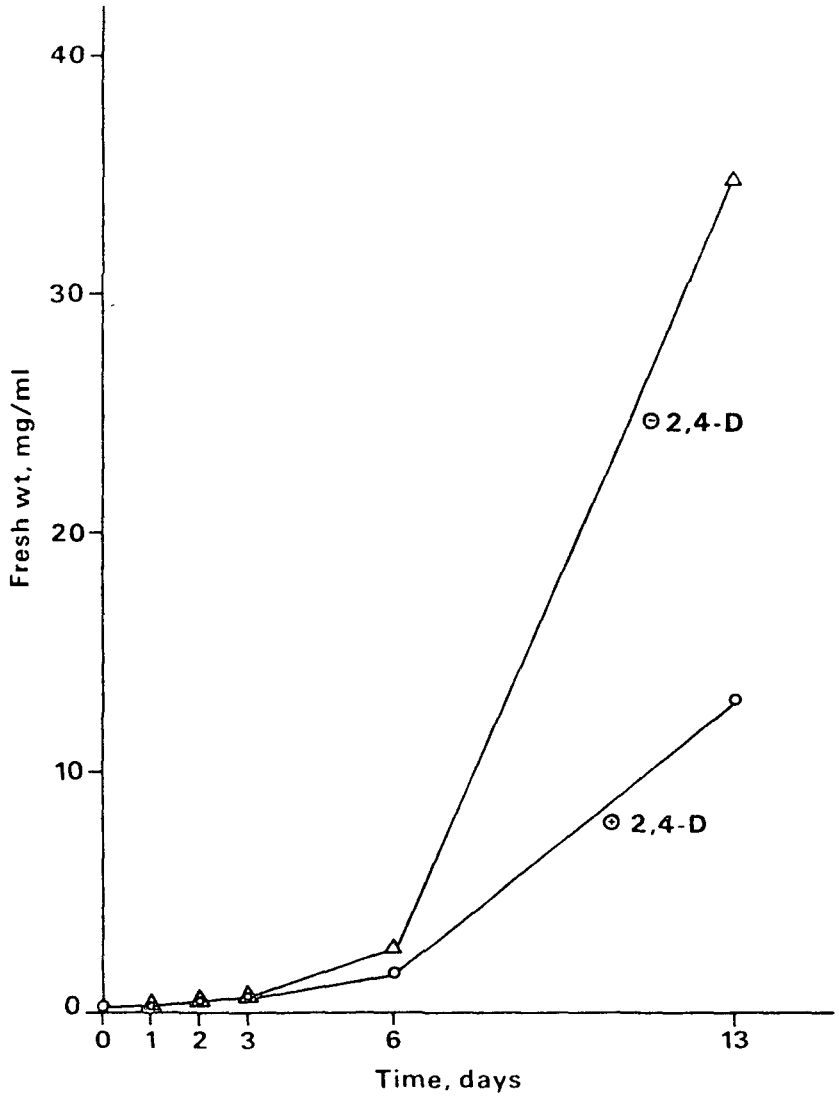
The general phenylpropanoid pathway. 1. Phenylalanine, 2. cinnamic acid, 3. p-coumaric acid, 4. caffeic acid, 5. ferulic acid, 6. a chalcone, 7. catechin. Enzymes: A. phenylalanine ammonia lyase, B. 4-cinnamic acid hydroxylase, C. p-coumaric acid 3-hydroxylase, D. O-methyltransferase.

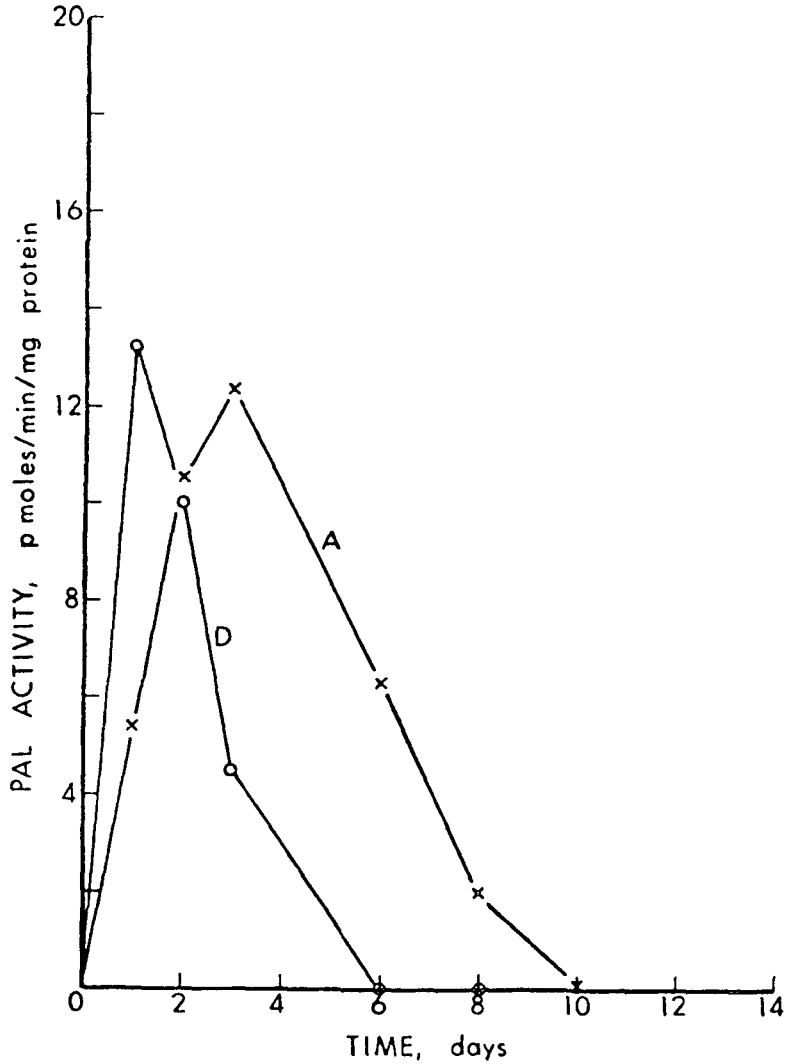


Tannin production in loblolly pine suspension cells under "embryogenic" (-) conditions (experiments A, B, C, D)

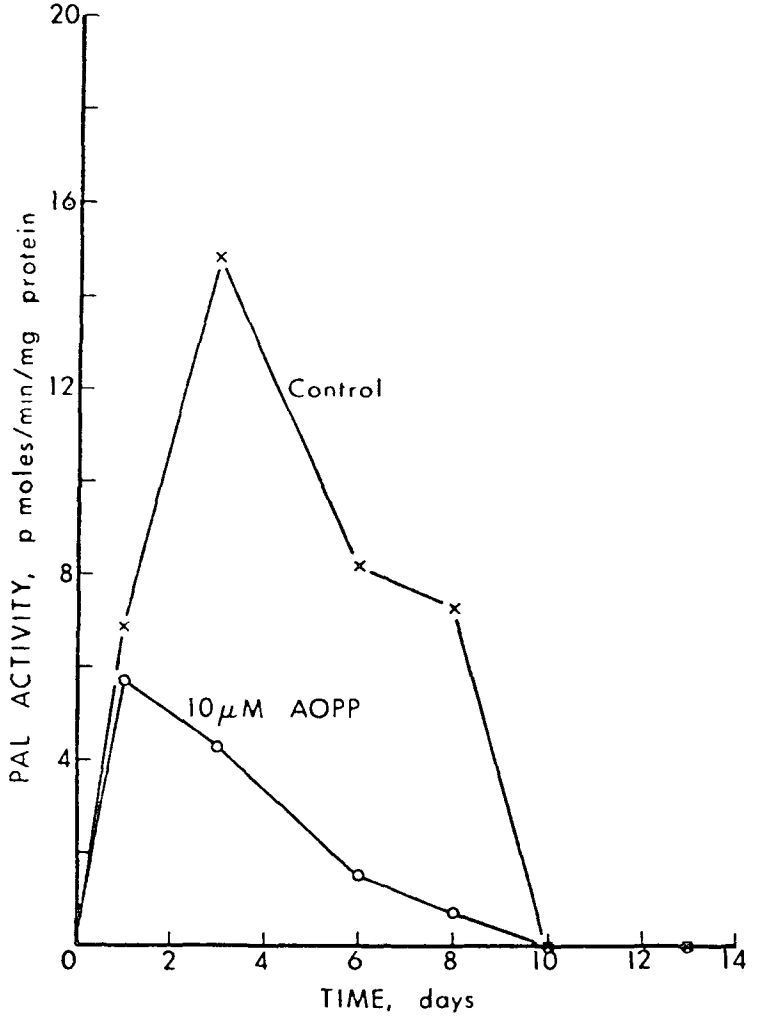


PAL activity in loblolly pine suspension cells under "embryogenic" (-) conditions (experiments A, B, C, D)

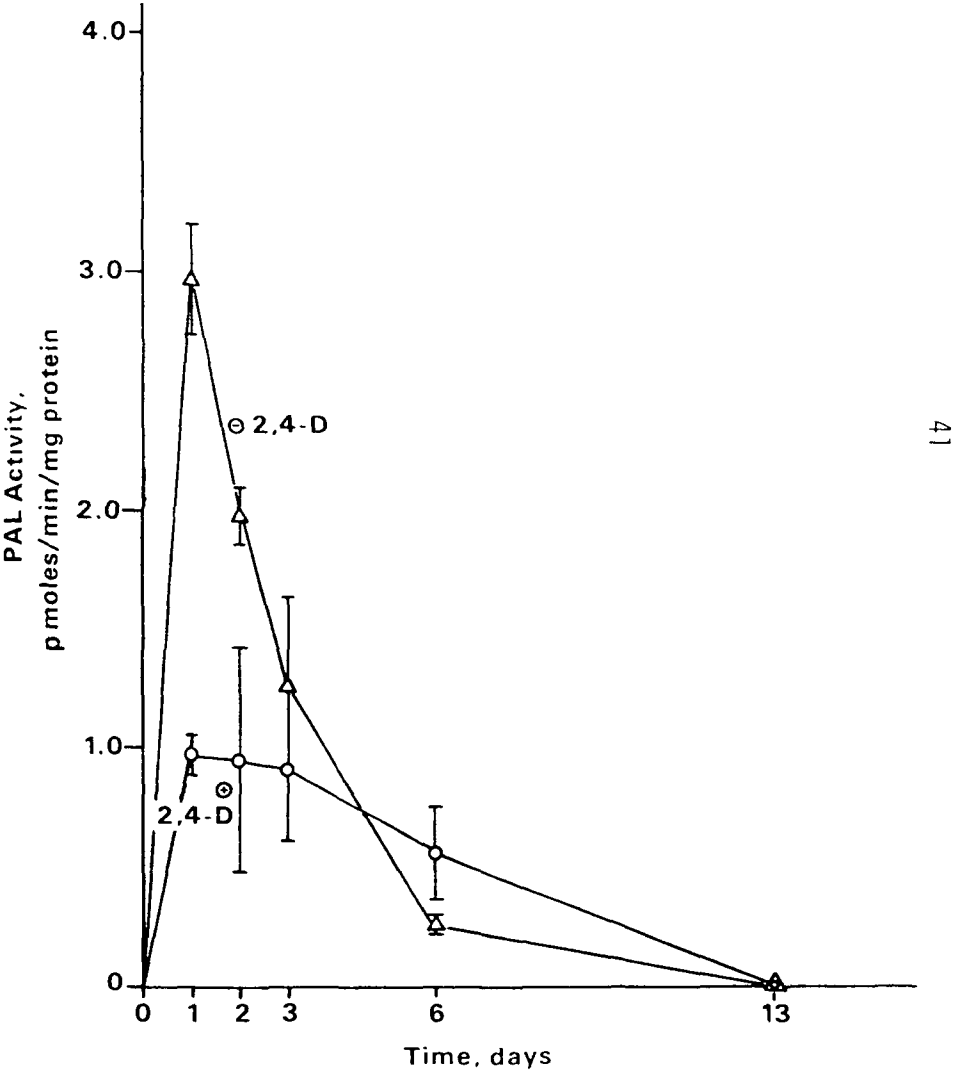


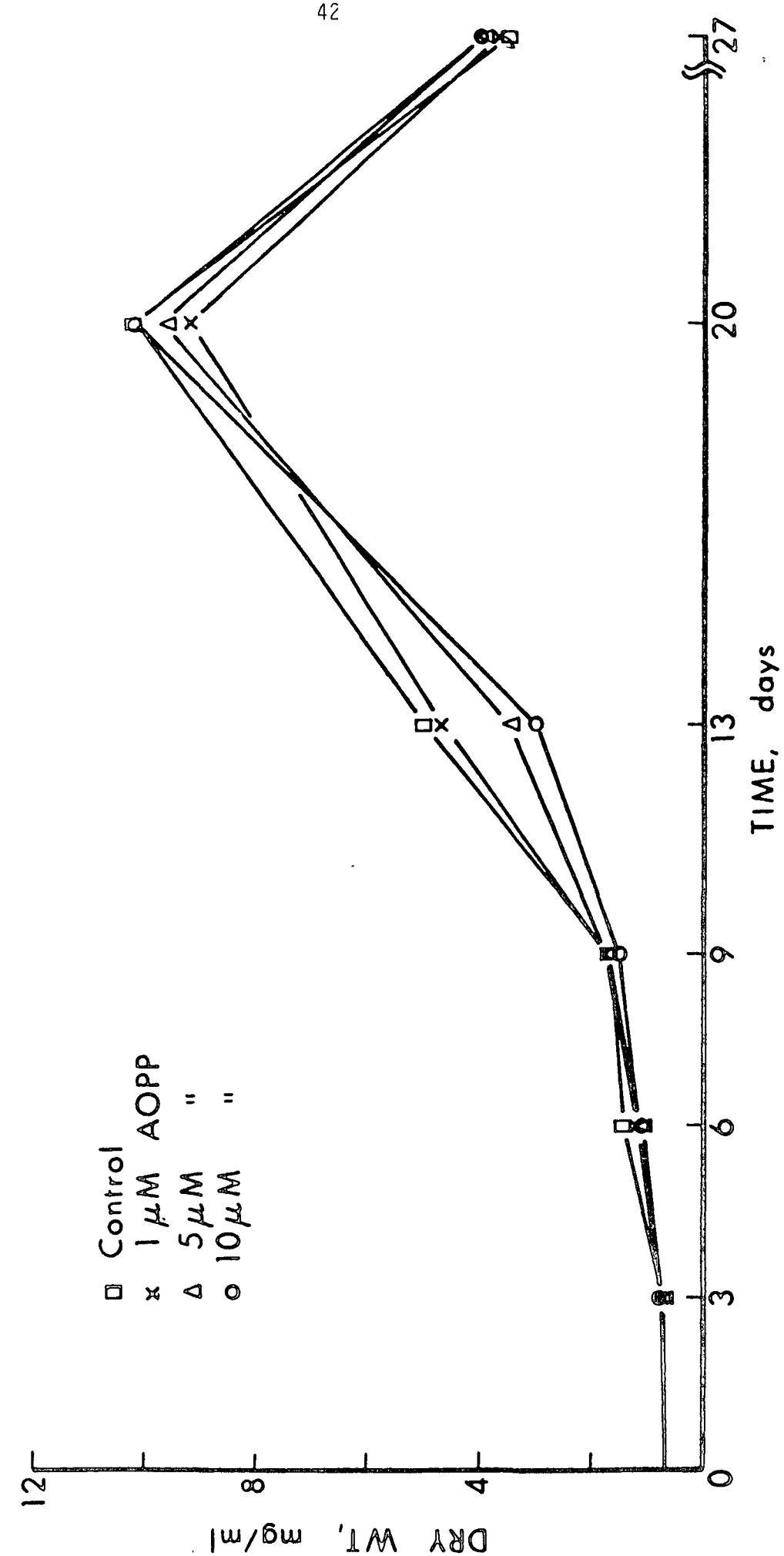


PAL activity in loblolly pine suspension cells under proliferative (+) conditions (experiments A, D)



Effect of AOPP on PAL activity of loblolly pine suspension cells in the absence of 2,4-D (experiment C)




DRY WT, mg/ml
12
8
4
0
3
6
9
13
20
27
TIME, days
□ Control
x 1 μM AOPP
Δ 5 μM "
o 10 μM "

Effects of phenolic compounds on the proliferative growth of wild carrot cells in the presence of 2,4-D.

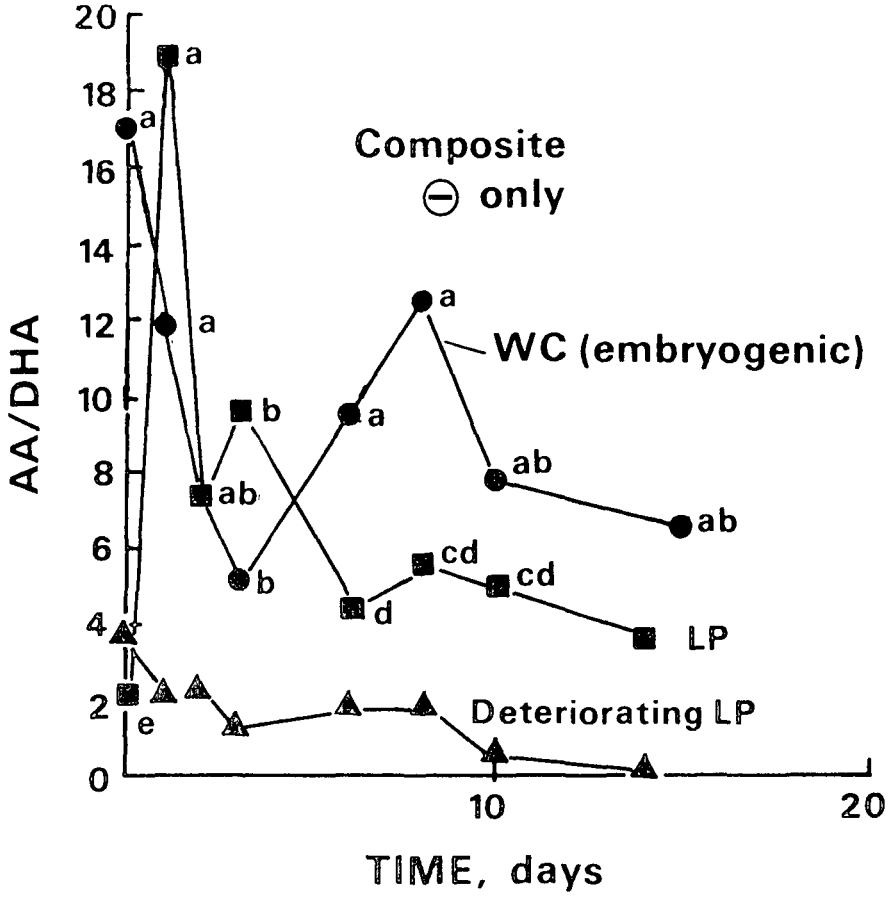
Compound Tested, M	Dry Weight, mg/mL/($\bar{x}$ ± SD)[b]				
	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
Caffeic acid	[a]3.4 ± 0.4[b]	4.8 ± 0.5[a]	4.7 ± 0.4[a]	4.9 ± 0.6[a]	4.7 ± 0.3[a]
Chlorogenic acid	[a]3.1 ± 0.3[c]	3.3 ± 0.4[c]	4.2 ± 0.2[b]	4.2 ± 0.6[b]	5.0 ± 0.2[a]
Cinnamic acid	1.8 ± 0.6[b]	4.5 ± 0.2[a]	4.6 ± 0.4[a]	4.5 ± 0.3[a]	4.6 ± 0.2[a]
p-Coumaric acid	2.5 ± 0.6[b]	4.6 ± 0.5[a]	4.2 ± 0.2[a]	4.4 ± 0.5[a]	4.5 ± 0.4[a]
Ferulic acid	3.6 ± 0.6[c]	4.6 ± 0.3[ab]	5.2 ± 0.5[a]	4.8 ± 0.4[ab]	5.1 ± 0.3[a]
D-Catechin	[a]3.5 ± 0.4[b]	4.0 ± 0.4[ab]	3.9 ± 0.3[ab]	4.2 ± 0.4[a]	3.5 ± 0.4[b]
Control, 0 M	4.3 ± 0.2				

[a]Browning observed.
[b]ANOVA and Duncan's Multiple Range Test were used to compare the treatment level means for each compound and with control; values with a common following superscript are not significantly different at the 95% confidence level. Control superscripts were: caffeic[a], chlorogenic[b], cinnamic[a], coumaric[a], ferulic[b], and catechin[a].

Effects of phenolic compounds on the embryogenic growth of wild carrot cells in the absence of 2,4-D.

Compound Tested, M	Embryos' Fresh weight, mg ($\bar{x}$ ± SD)[c]				
	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
Caffeic acid	[a]396 ± 109[a]	389 ± 54[a]	375 ± 39[a]	436 ± 81[a]	355 ± 83[a]
Chlorogenic acid	[ab]159 ± 29[c]	[a]344 ± 83[ab]	313 ± 30[b]	342 ± 42[ab]	342 ± 50[ab]
Cinnamic acid	[b]110 ± 55[b]	404 ± 22[a]	321 ± 86[a]	322 ± 81[a]	364 ± 82[a]
p-Coumaric acid	289 ± 79[d]	406 ± 26[a]	365 ± 71[abc]	313 ± 48[bcd]	309 ± 28[cd]
Ferulic acid	397 ± 82[ab]	[b]434 ± 37[a]	[b]320 ± 82[bc]	[b]328 ± 62[bc]	[b]276 ± 79[c]
D-Catechin	[ab]361 ± 35[ab]	[b]377 ± 38[ab]	[b]345 ± 58[ab]	[b]343 ± 42[ab]	[b]315 ± 56[b]
Control, 0 M	385 ± 34				

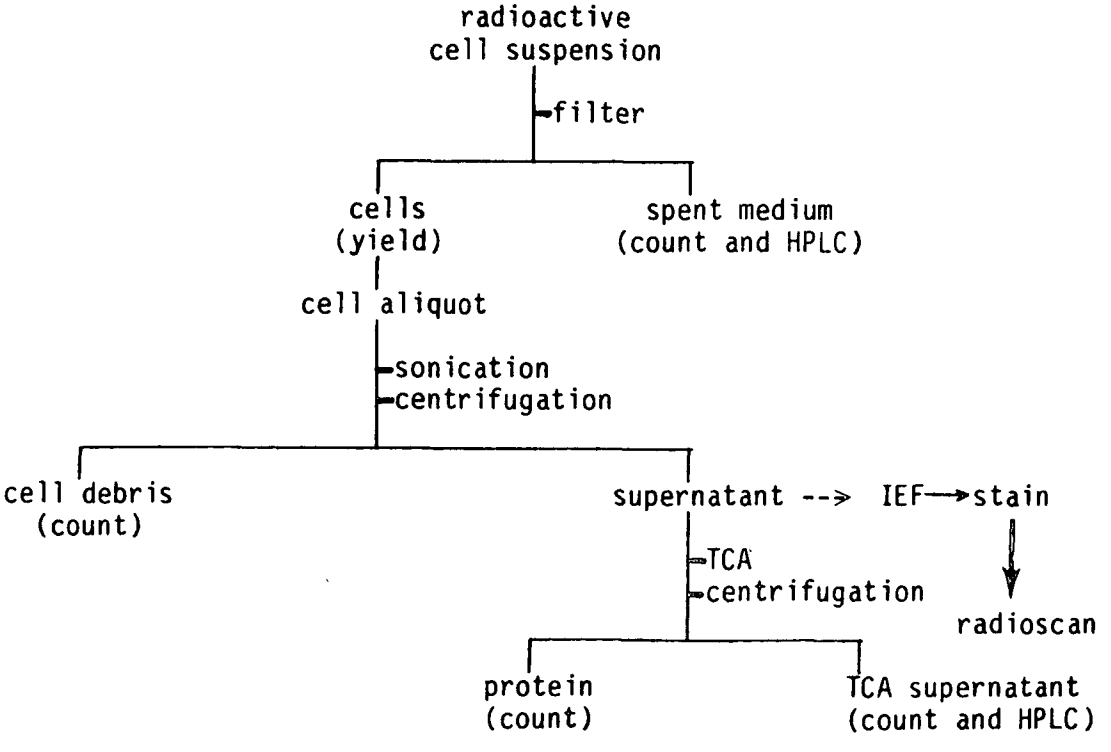
[a]Browning observed.
[b]Greening observed.
[c]ANOVA and Duncan's Multiple Range Test were used to compare the treatment level means for each compound and with control; values with a common following superscript are not significantly different at the 95% confidence level. Control superscripts were: caffeic[a], chlorogenic[a], cinnamic[a], coumaric[ab], ferulic[ab], and catechin[a].

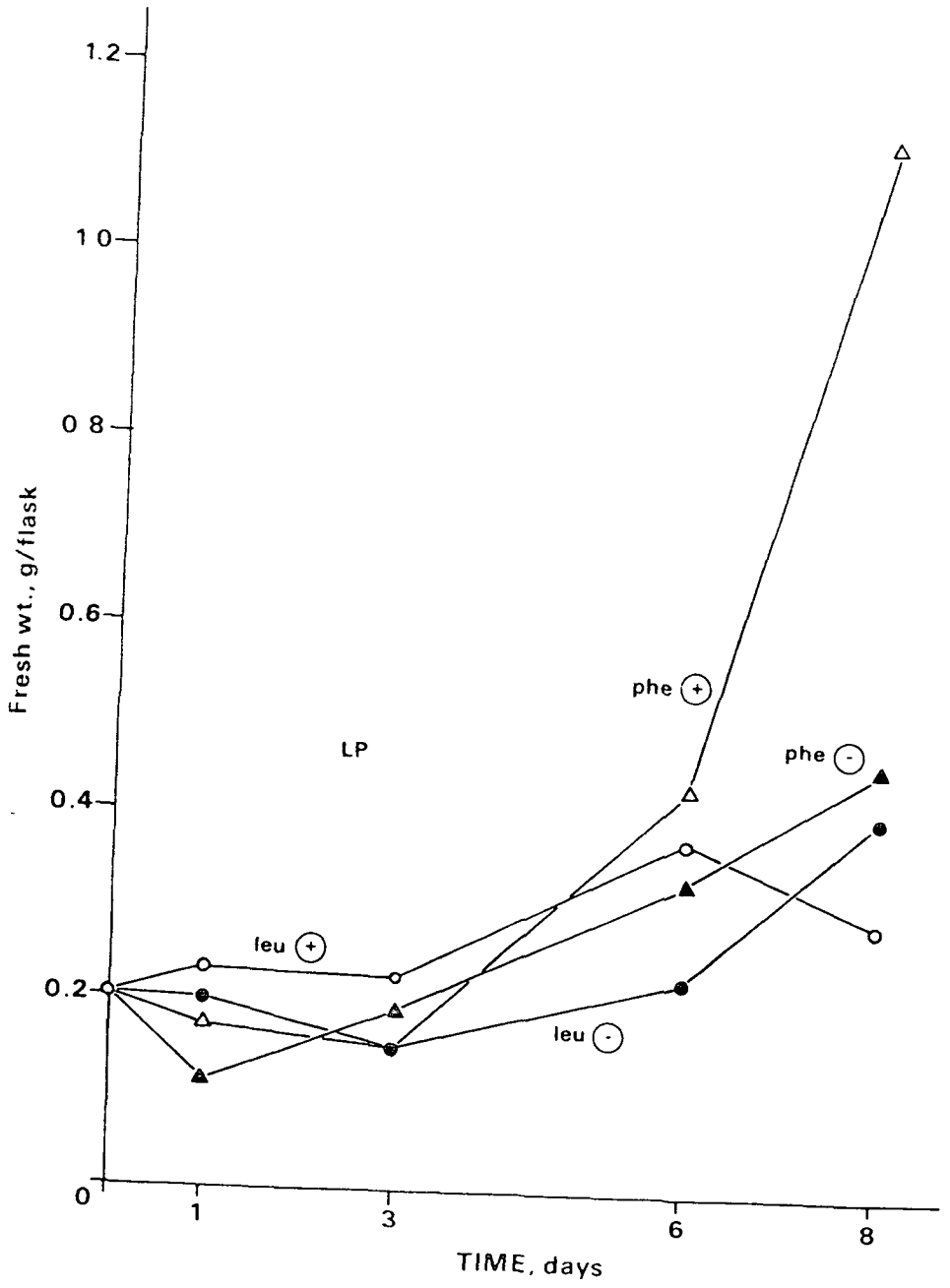

20
18
16
14
12
10
8
6
4
2
0
AA/DHA
Composite
⊖ only
WC (embryogenic)
LP
Deteriorating LP
a
a
a
b
ab
b
a
a
ab
ab
cd
cd
d
e
10
20
TIME, days

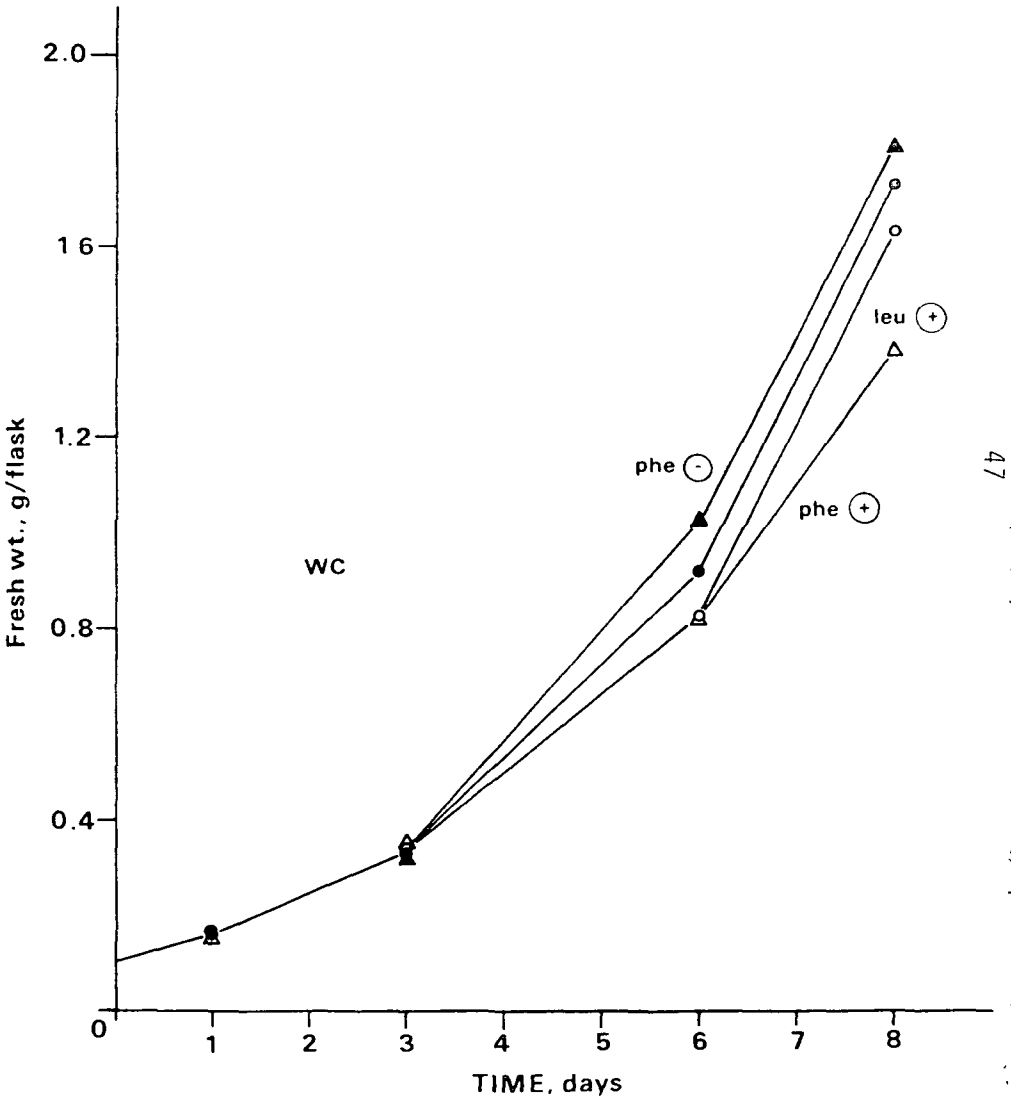
TRACER STUDIES

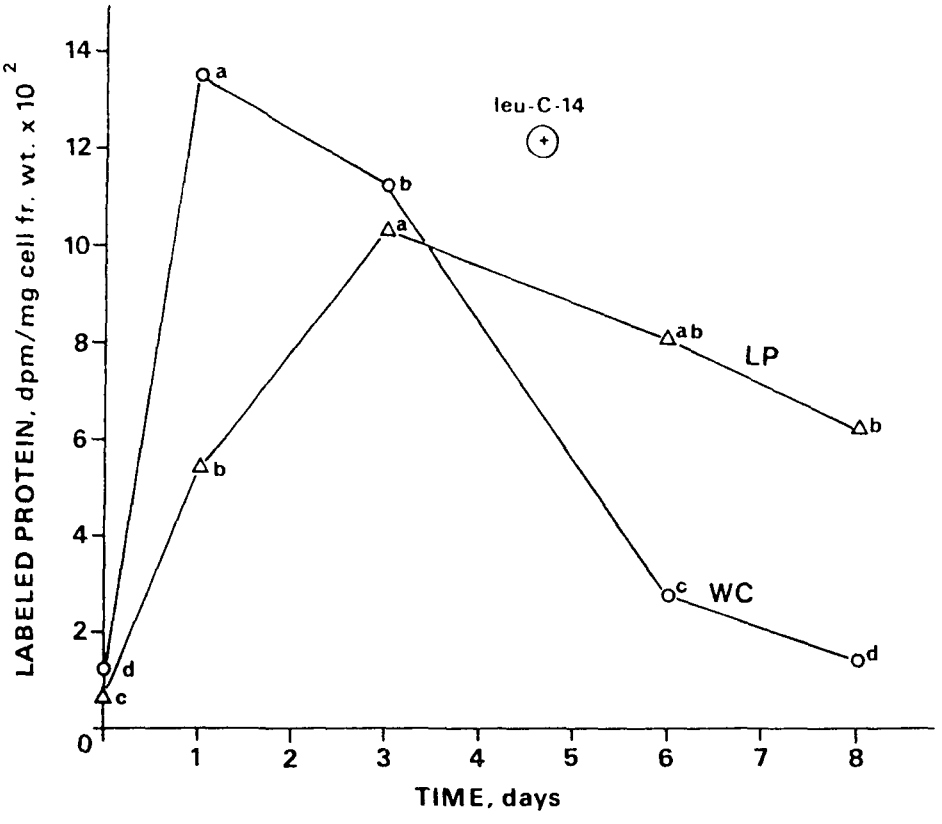
Q. Since PAL activity appears to be stronger and more persistent in cultured pine cells than in cultured wild carrot cells, could it be that the greater drain on phenylalanine in pine cells is having undesirable effects on protein synthesis? What are the relative fates of leucine (primarily a protein precursor) and phenylalanine (precursor of both protein and phenolics) in pine versus carrot cells in culture?

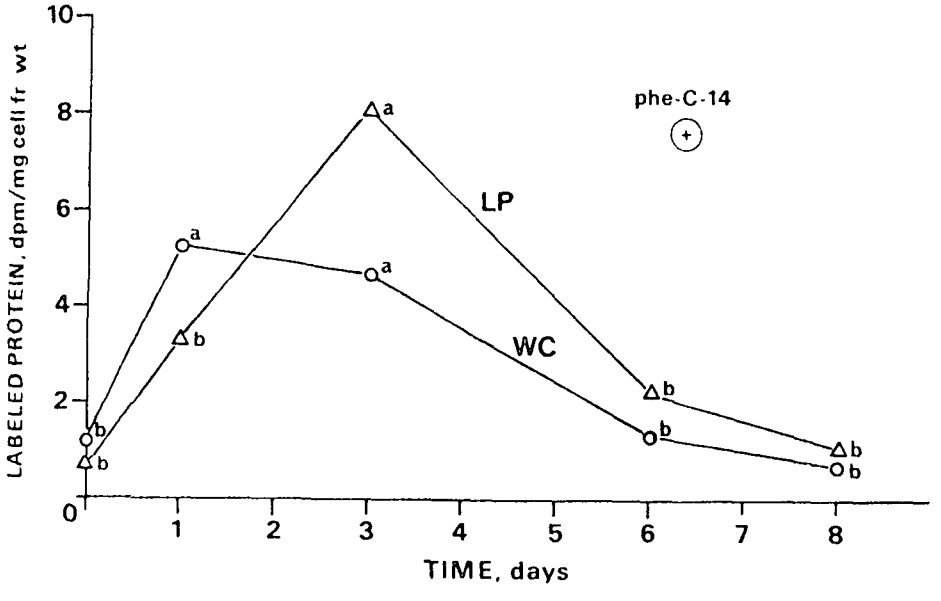
A. While experi ments with pine versus carrot suspension cells using radioactive leu and phe did not indicate severe problems with protein synthesis in the pine cells, potentially useful differences in the tracer results for the two species in culture will be described. Among the notable findings was evidence that protein degradation is a significant feature in launched pine cells relative to launched carrot cells or proliferative cells of either species

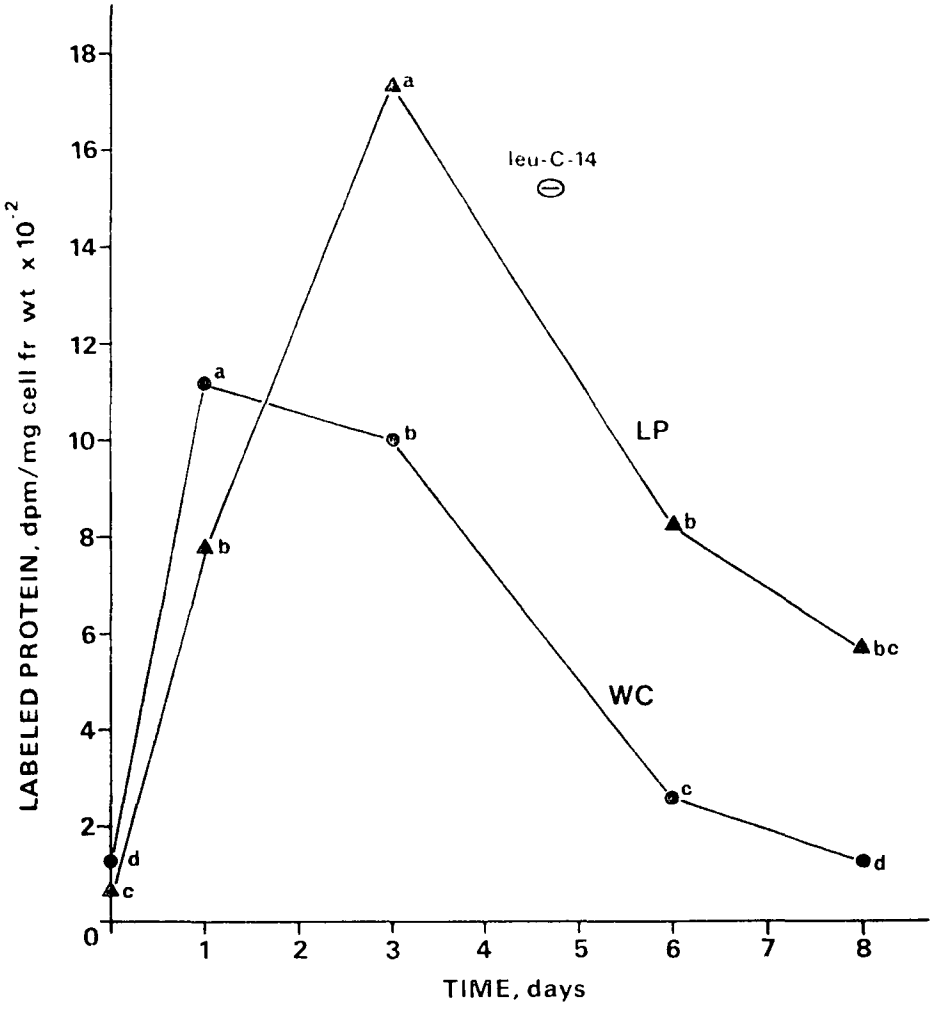


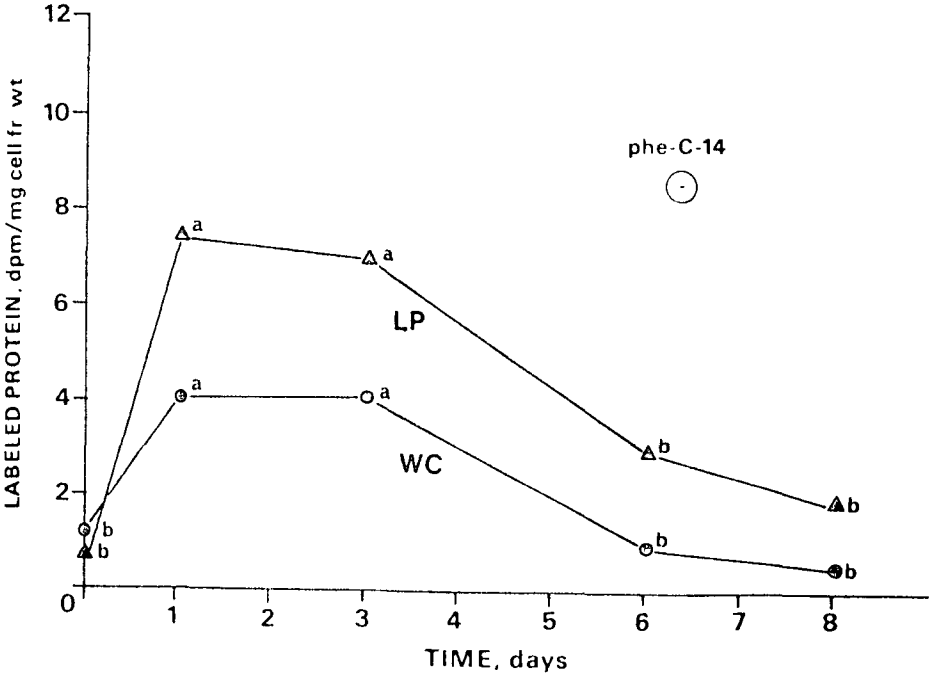

1.2
1.0
0.8
0.6
0.4
0.2
0
Fresh wt., g/flask
LP
phe +
phe -
leu +
leu -
1
3
6
8
TIME, days

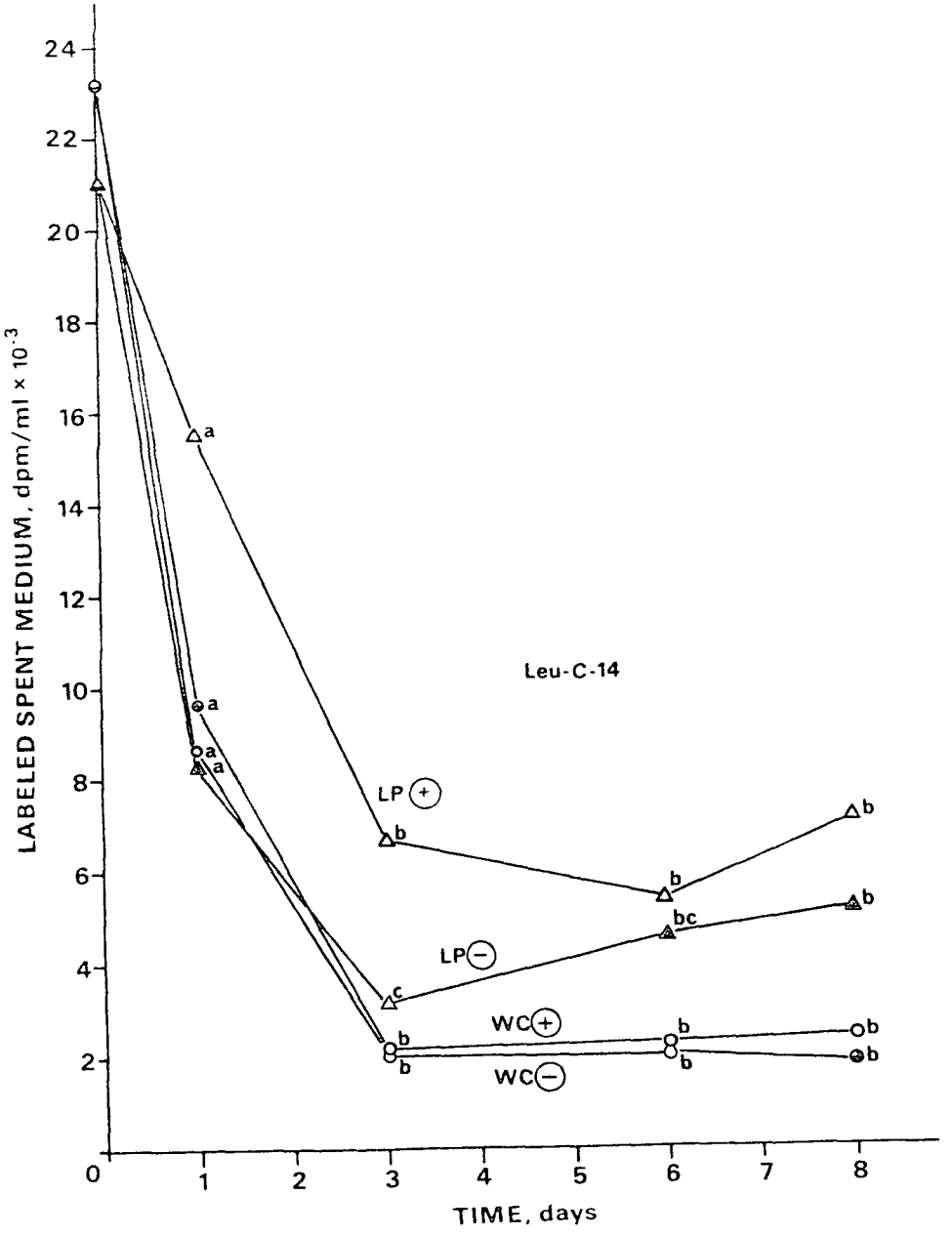

2.0
1.6
1.2
0.8
0.4
0
Fresh wt., g/flask
WC
phe -
phe +
leu +
1
2
3
4
5
6
7
8
TIME, days

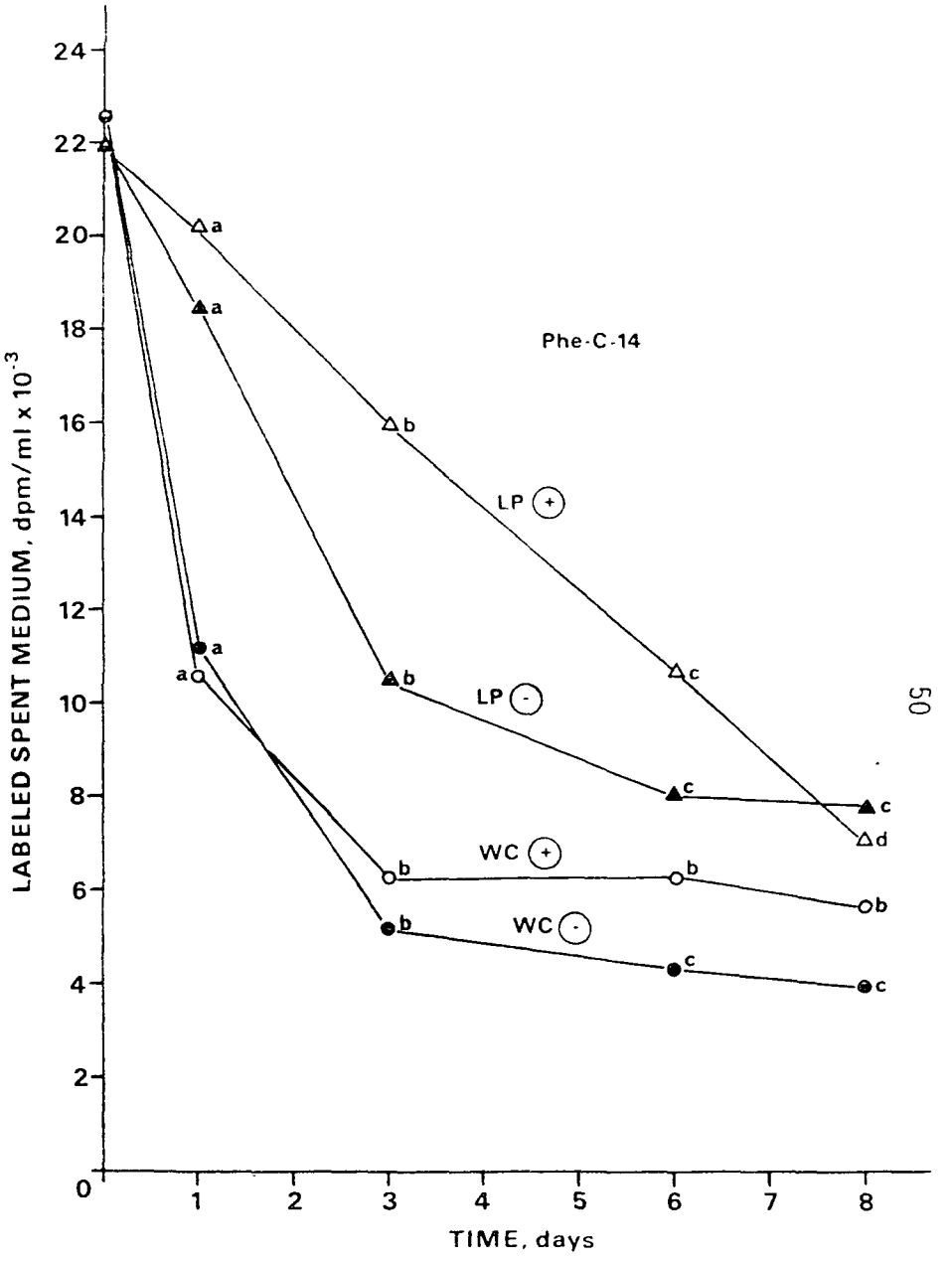

LABELED PROTEIN, dpm/mg cell fr. wt. x 10 2
leu-C-14
LP
WC
TIME, days

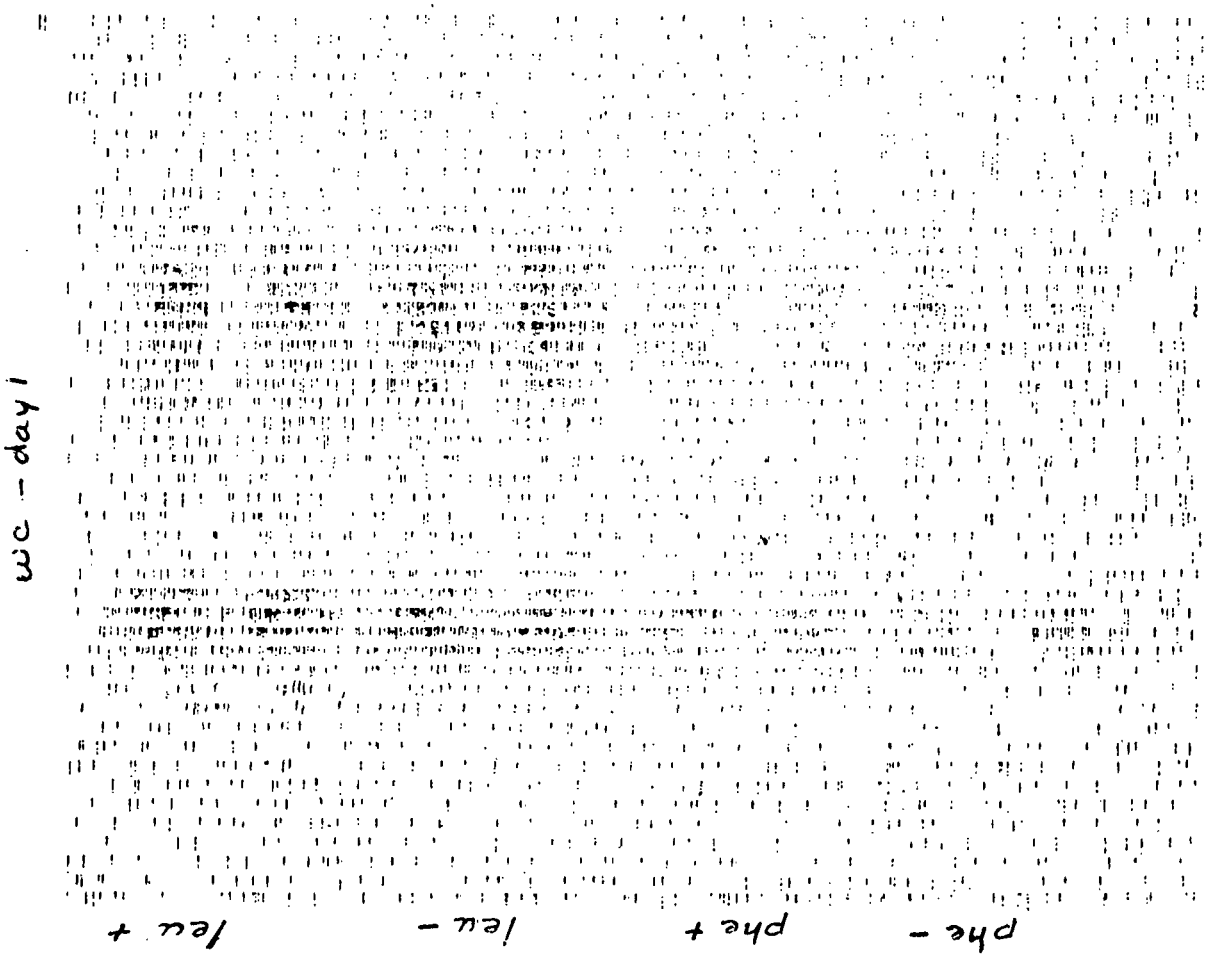

LABELED PROTEIN, dpm/mg cell fr wt
phe-C-14
LP
WC
TIME, days

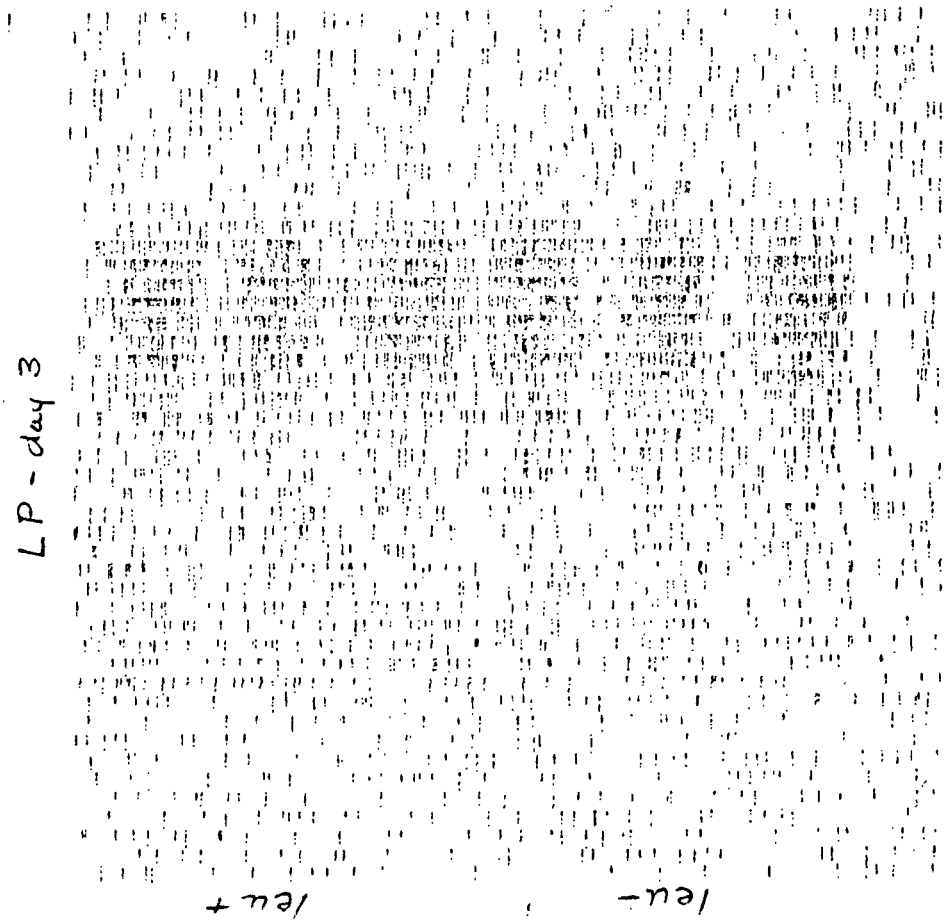

LABELED PROTEIN, dpm/mg cell fr wt x 10^{-2}
18
16
14
12
10
8
6
4
2
0
leu-C-14
LP
WC
a
b
c
d
bc
1
2
3
4
5
6
7
8
TIME, days

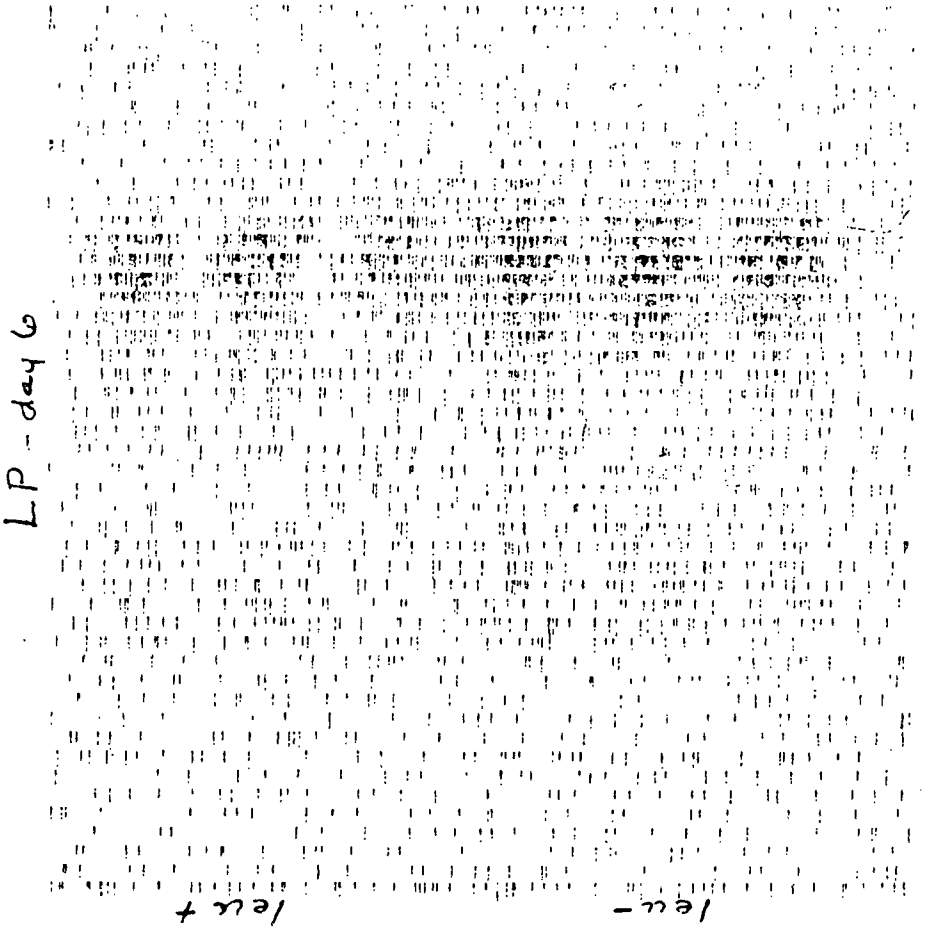

LABELED PROTEIN, dpm/mg cell fr wt
12
10
8
6
4
2
0
phe-C-14
LP
WC
a
b
1
2
3
4
5
6
7
8
TIME, days

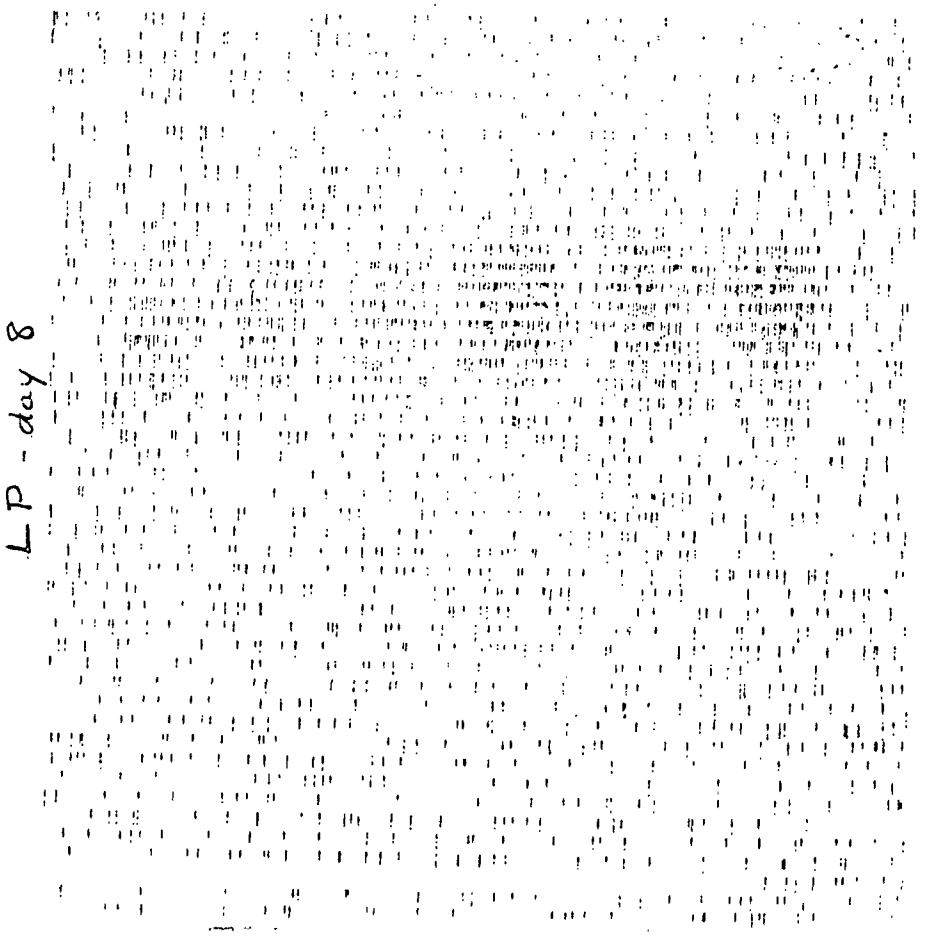

LABELED SPENT MEDIUM, dpm/ml x 10⁻³
TIME, days
Leu-C-14
LP (+)
LP (−)
WC (+)
WC (−)

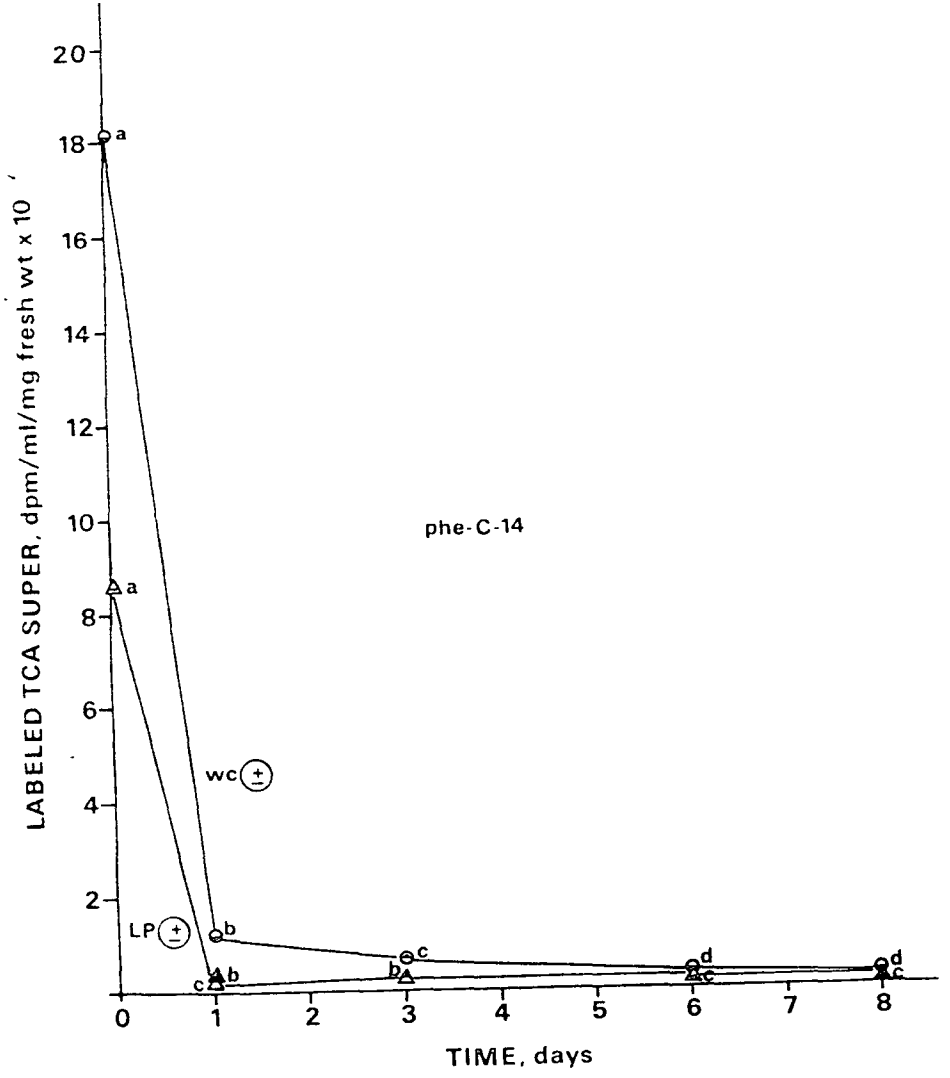

LABELED SPENT MEDIUM, dpm/ml x 10⁻³
TIME, days
Phe-C-14
LP (+)
LP (-)
WC (+)
WC (-)

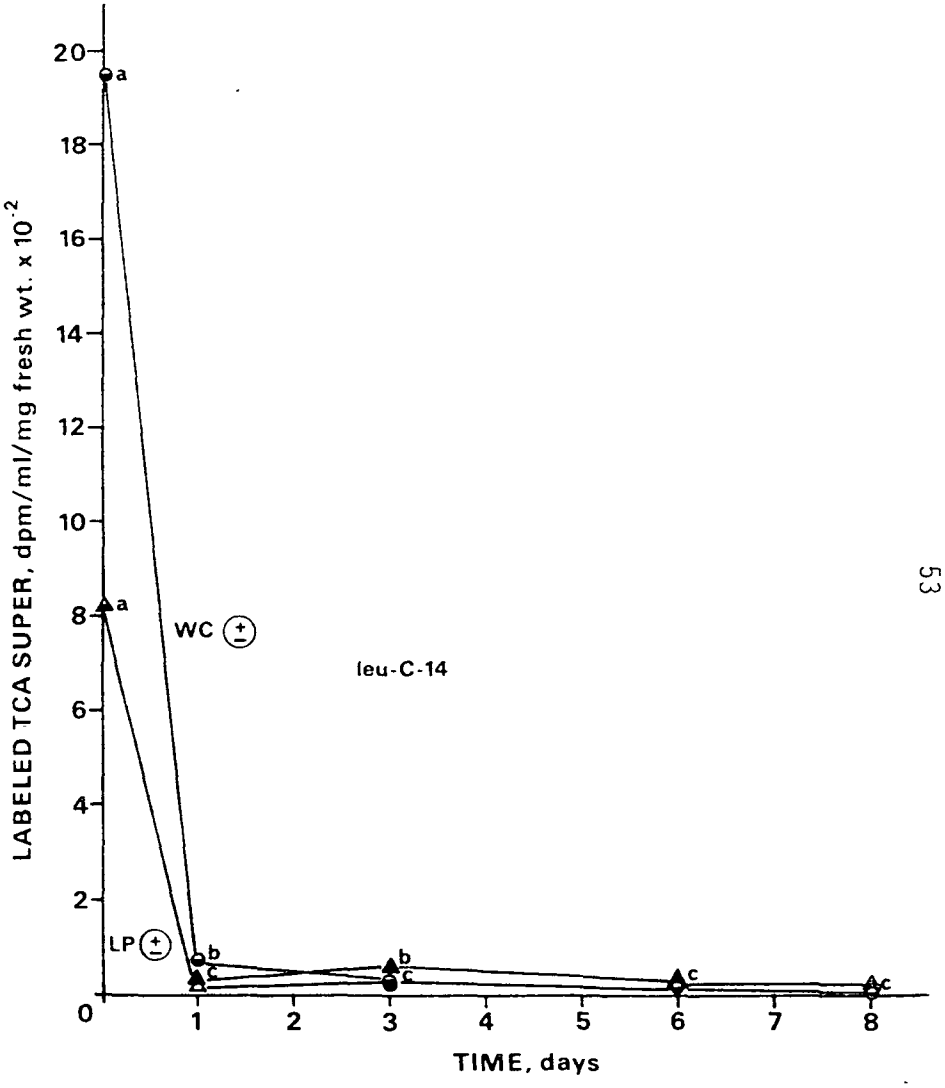

WC - day 1
phe -
phe +
leu -
leu +


LP - day 3
leu -
leu +


LP - day 6
leu -
leu +


LP - day 8


LABELED TCA SUPER, dpm/ml/mg fresh wt x 10
20
18
16
14
12
10
8
6
4
2
0
1
2
3
4
5
6
7
8
TIME, days
phe-C-14
wc ±
LP ±
a
b
c
d


LABELED TCA SUPER, dpm/ml/mg fresh wt. x 10^{-2}
20
18
16
14
12
10
8
6
4
2
0
1
2
3
4
5
6
7
8
TIME, days
leu-C-14
WC ±
LP ±
a
b
c

HYPOXYLON MAMMATUM AS A WOUND PARASITE

1. Role of wound producing agents (insects, environmental).
2. Fungicidal activity of aspen bark.

ADVANTAGES OF IN VITRO SCREENING FOR DISEASE RESISTANCE

1. Saving of space.
2. Saving of time.
3. Elimination of variation induced by the environment.

EXAMPLES OF TREES FROM CELLS

1. Populus tremuloides (Winton, 1970): See South Krannert lawn.
2. Populus euramericana (Lester and Berbee, 1977): 38% of plants were variant.
3. Coffea arabica (Sondahl, 1984): High frequency somatic embryogenesis unsuitable for clonal propagation.

SUMMARY OF IN VITRO SCREENING FOR DISEASE RESISTANCE IN WOODY SPECIES

Tissue Culture

1. Embryos of Pinus lambertiana inoculated with Cronartium ribicola (Diner and Mott, 1982).
2. Embryos of Pinus taeda L. inoculated with Cronartium quercuum (Gray and Amerson, 1983).
3. Leaf discs of Populus deltoides inoculated with Melamspora medusae (Shain and Jarlfors, 1984).

Cell Culture

1. Callus cultures of P. lambertiana inoculated with Cronartium ribicola (Diner, et al., 1984).
2. Suspension cultures of Ulmus americana inoculated with Ceroeystis ulmi (Hindal and McNabb, 1973).
3. Callus cultures of Castanea dentata with Endothia parasitica (Hebard, et al., 1978).
4. Cultures of Pseudotsuga menziesii inoculated with Cronartium ribicola (Harvey and Grasham, 1971).

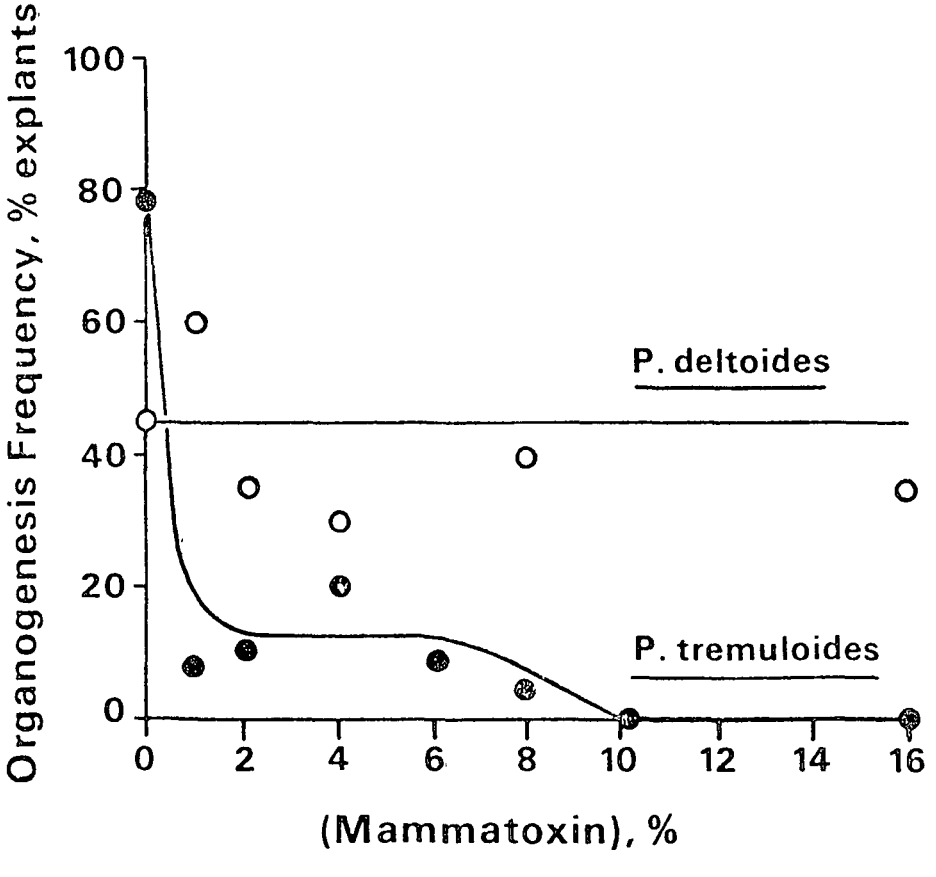
OBJECTIVES

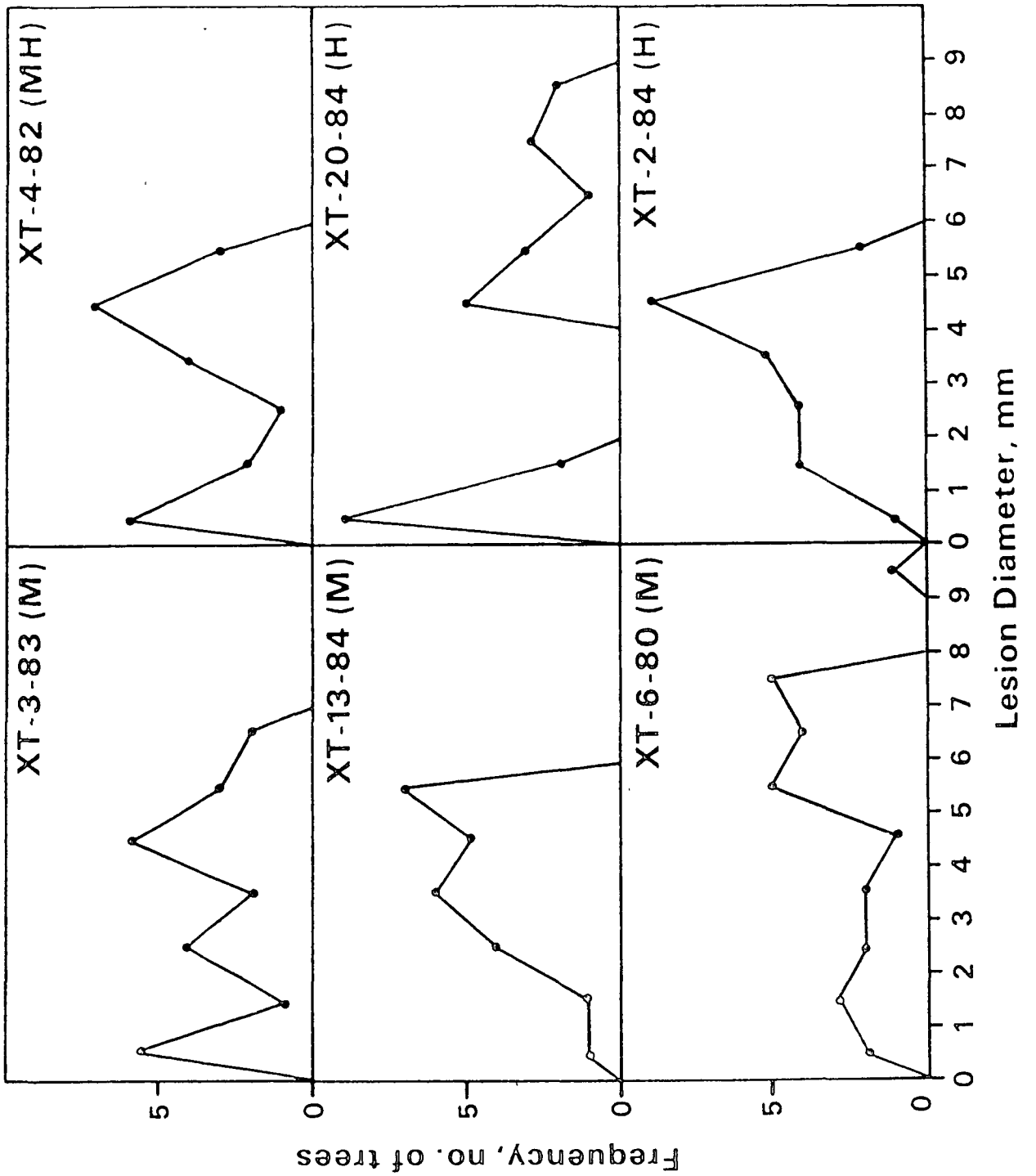
1. Employ mammatoxin in a tissue culture system to obtain toxin-resistant plants.
    a. Develop a micropropagation method from seedling explants of P. tremuloides.
2. Determine origin of resistance response.
    a. Is the micropropagation system clonal or subject to variation?

EFFECT OF MEDIA AND GROWTH REGULATOR REGIME ON SHOOT PROLIFERATION CHARACTERISTICS OF SEEDLING EXPLANTS (XT-3-83)

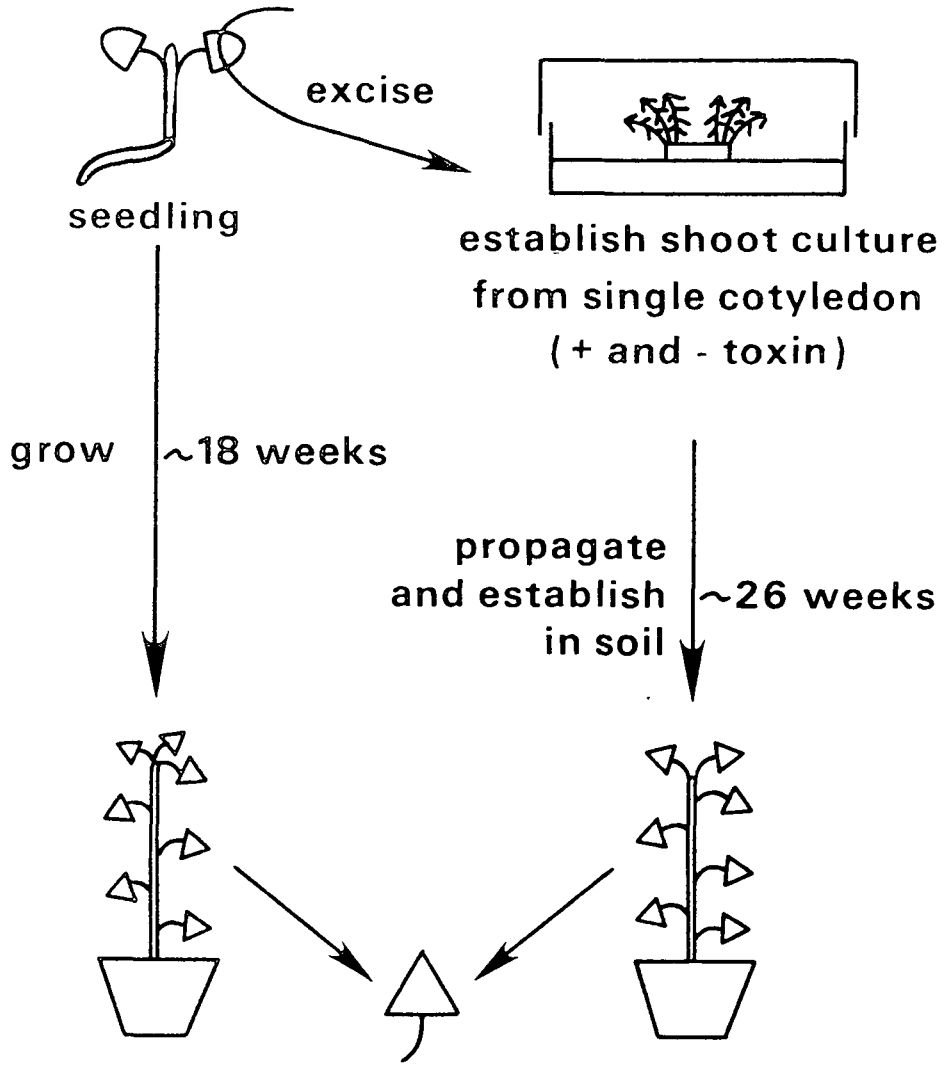
Medium	BA/NAA, mg/L	Ratio (BA/NAA)	Shoot induction,* % Hypocotyls	Shoot induction,* % Cotyledons	Shoots/explant Hypocotyls	Shoots/explant Cotyledons
MS	1.0/0.01	100:1	95^{a}	47^{b}	6.1^{b}	4.4^{a}
	1.0/0.1	10:1	96^{a}	80^{a}	5.9^{b}	4.8^{a}
	1.0/1.0	1:1	61^{b}	48^{b}	3.1^{c}	3.1^{b}
	0.1/0.01	10:1	65^{b}	29^{c}	1.5^{d}	1.7^{b,c}
	0.1/0.1	1:1	50^{b}	15^{c,d,e}	1.1^{d}	2.0^{b,c}
LM	1.0/0.01	100:1	84^{a}	20^{e,d}	6.8	5.0^{a}
	1.0/0.1	10:1	95^{a}	19^{c,d}	7.3^{a}	4.4^{a}
	1.0/1.0	1:1	31^{c}	4.6^{d,e}	3.5^{c}	1.4^{b,c}
	0.1/0.01	10:1	66^{c}	5.4^{d,e}	1.3^{d}	1.6^{b,c}
	0.1/0.1	1:1	32^{c}	0.86^{e}	1.0^{d}	0.14^{c}

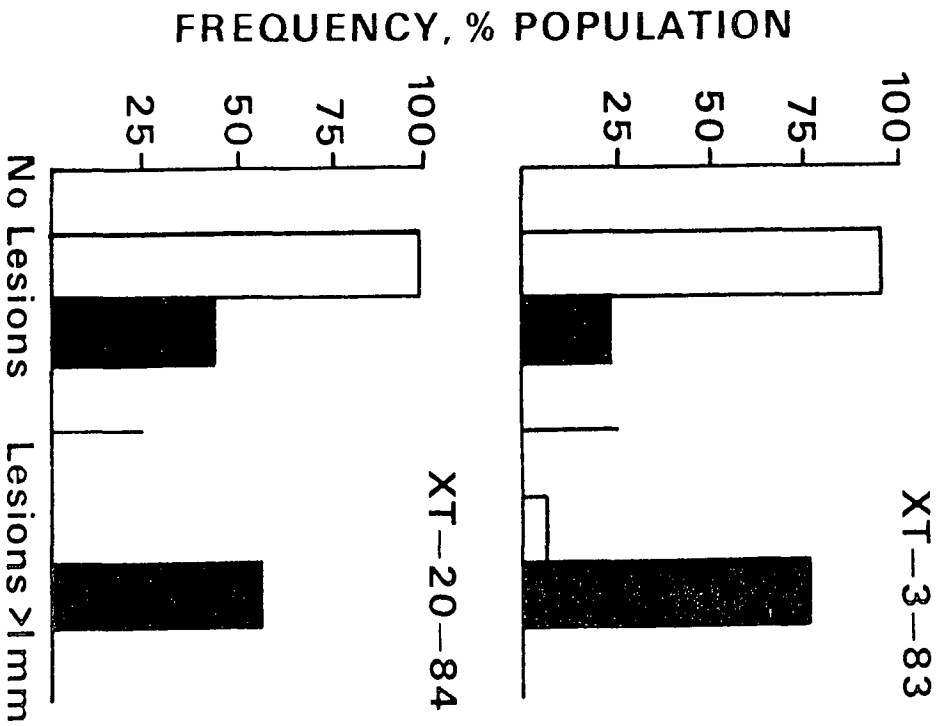
* Means followed by a common superscript within a column are not significantly different by Duncan's Multiple Range Test.





Frequency of Mammatoxin Response as a Function of Lesion Diameter for Six Full-Sib P. tremuloides Crosses (25 seedlings/cross; value in parentheses indicates IPC Project 3250 hypoxylon rating system).


excise
seedling
establish shoot culture
from single cotyledon
(+ and - toxin)
grow
~18 weeks
propagate
and establish
in soil
~26 weeks
compare bioassay of ortet (seedling)
vs. ramet (tissue culture propagule)



Mammatoxin Response of a Second-Order Seedling Population Derived by the In Vitro Bioassay (open bars = second-order population; closed bars = control population).

COMPARISON OF CANKER SUSCEPTIBILITY TO MAMMATOXIN RESPONSE FOR VARIOUS PROGENY GROUPS

Cross	Susceptibility	Toxin Resistance, %
XT-6-80	Medium	24
XT-3-83	Medium	28
XT-13-84	Medium	8
XT-4-82	Medium-high	32
XT-2-84	High	12
XT-20-84	High	44

INITIATION OF CELL LINES

Hypothesis: Use of cell lines initiated from immature embryos will increase the chances of obtaining somatic

embryogenesis

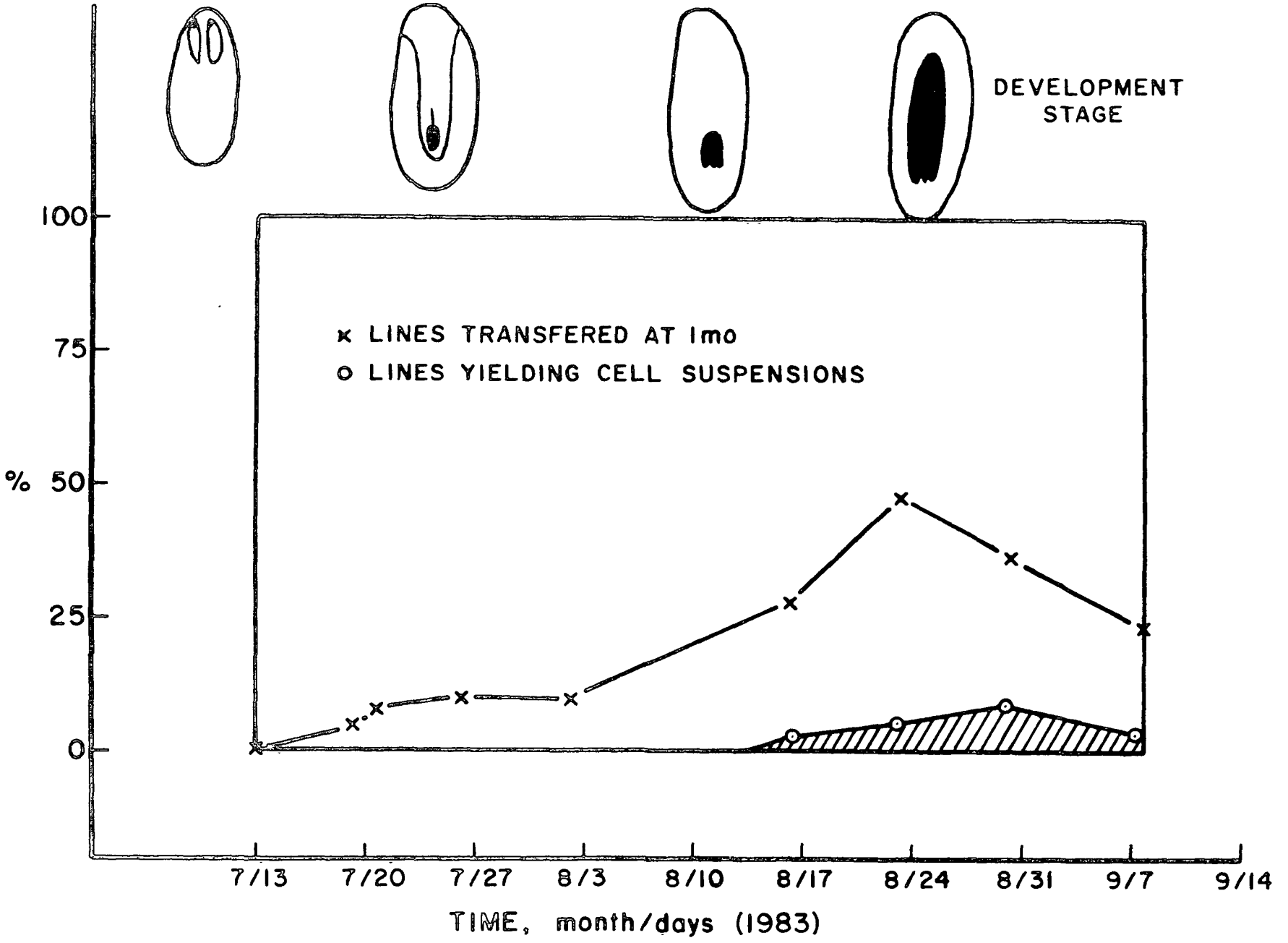
Objectives: (1) Obtain high quality cell lines

(2) Develop a near optimum initiation medium

(3) Establish a protocol for routinely initiating new cell lines from immature embryos

CELL LINE INITIATION SUCCESS

- Varied significantly with media
- Varied significantly with collection dates
- Was significantly greater in the dark than in the light (on agar)
- Was greater on agar than in liquid



Percent cell lines successfully obtained in 1983 from loblolly pine immature embryos.

SYNTHETIC AUXIN TRIALS

Question: Are there any synthetic auxins which stimulate proliferative growth of pine cell suspensions better than 2,4-D?

Answer: Yes. In our synthetic auxin trials we found 2 forms of NAA stimulated more growth than 2,4-D.

Table 1. Effect of various synthetic auxins on proliferative growth of pine cell suspensions.

Compound Tested, mg/L	Day 14 Line F2 Cell Growth, mean dry wt. (mg/mL)[a]						
	0[b]	0.001	0.01	0.1	1.0	2.0 (2,4-D)[b]	10.0
2,4,5-T	1.6^{b}	2.0^{ab}	1.8^{ab}	1.8^{ab}	2.5^{ab}	2.9^{a}	1.4^{b}
1-NAA	1.6^{d}	2.0^{cd}	2.5^{cd}	4.3^{b}	7.5^{a}	2.9^{c}	0.3^{e}
2-NAA	1.6^{bc}	1.7^{bc}	2.0^{b}	1.8^{bc}	3.4^{a}	2.9^{ab}	0.5^{c}
3 IAA-Asp	1.6^{bc}	1.3^{bc}	1.1^{c}	2.5^{ab}	1.4^{bc}	2.9^{a}	0.9^{c}
2,4,6-T	1.6^{bc}	1.6^{bc}	2.7^{ab}	2.4^{ab}	2.0^{abc}	2.9^{a}	0.8^{c}
POA	1.6^{b}	2.3^{ab}	1.8^{ab}	2.0^{ab}	1.4^{b}	2.9^{a}	1.5^{b}
2-PP	1.6^{b}	1.1^{b}	1.1^{b}	1.2^{b}	1.4^{b}	2.9^{a}	1.3^{b}

[a]ANOVA and Duncan's Multiple Range Test were run for mean dry weight differences. Values with a common superscript within a row are not significantly different (P = .05).

[b]Control mean.

Table 2. Effect of various synthetic auxins on proliferative growth of pine cell suspensions.

Compound Tested, mg/L	Day 14 Line F2 Cell Growth, mean dry wt. (mg/mL)[a]							
	0[b]	0.001	0.01	0.1	1.0	2.0	(2,4-D)[b]	10.0
PA	1.4[ab]	0.7[abc]	0.3[c]	0.8[abc]	0.8[abc]		1.4[a]	0.8[abc]
Trpol	1.4[ab]	1.2[ab]	0.8[ab]	0.6[ab]	0.6[ab]		1.4[a]	1.1[ab]
CHAA	1.4[a]	1.4[a]	1.7[a]	1.5[a]	1.9[a]		1.4[a]	1.2[a]
SB-13	1.4[ab]	1.0[b]	1.3[ab]	0.8[b]	1.8[a]		1.4[ab]	1.4[ab]
IBA	1.4[a]	1.6[a]	1.2[a]	1.2[a]	1.2[a]		1.4[a]	1.3[a]

[a]ANOVA and Duncan's Multiple Range Test were run for mean dry weight differences. Values with a common superscript within a row are not significantly different (P = .05).

[b]Control mean.

Table 3. Effect of various synthetic auxins on proliferative growth of pine cell suspensions.

Compound Tested, mg/L	Day 14 Line F2 Cell Growth, mean dry wt. (mg/mL)[a]							
	0[b]	0.001	0.01	0.1	1.0	2.0	(2,4-D)[b]	10.0
3-PP	1.6[a]	1.6[a]	1.5[a]	1.6[a]	1.5[a]		1.8[a]	1.8[a]
BA	1.6[a]	1.5[a]	1.6[a]	1.5[a]	1.5[a]		1.8[a]	1.7[a]
Trp	1.6[ab]	1.7[ab]	1.4[ab]	1.6[ab]	1.3[b]		1.8[a]	1.7[ab]

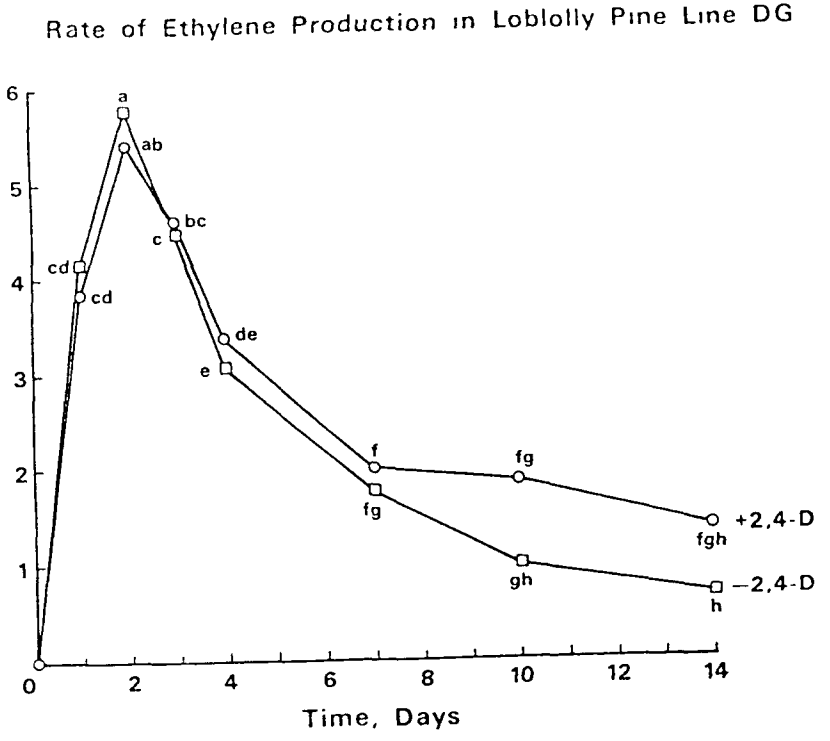
[a]ANOVA and Duncan's Multiple Range Test were run for mean dry weight differences. Values with a common superscript within a row are not significantly different (P = .05).

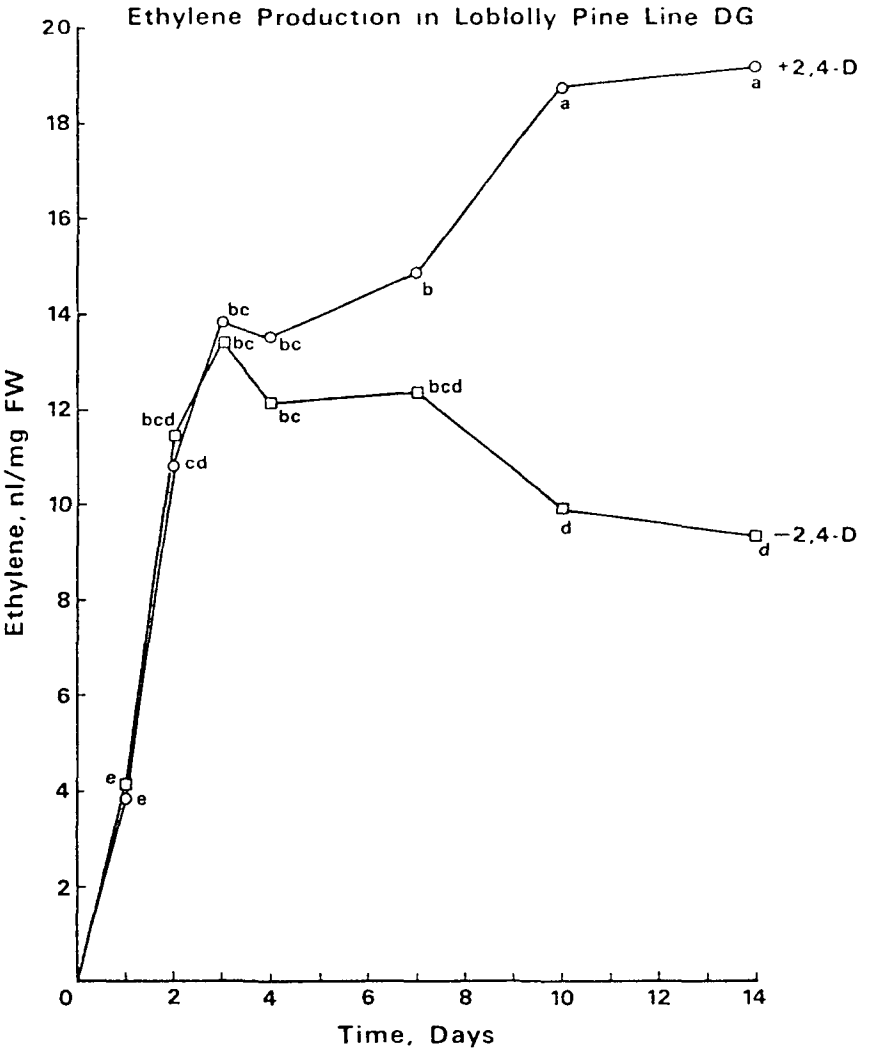
[b]Control mean.

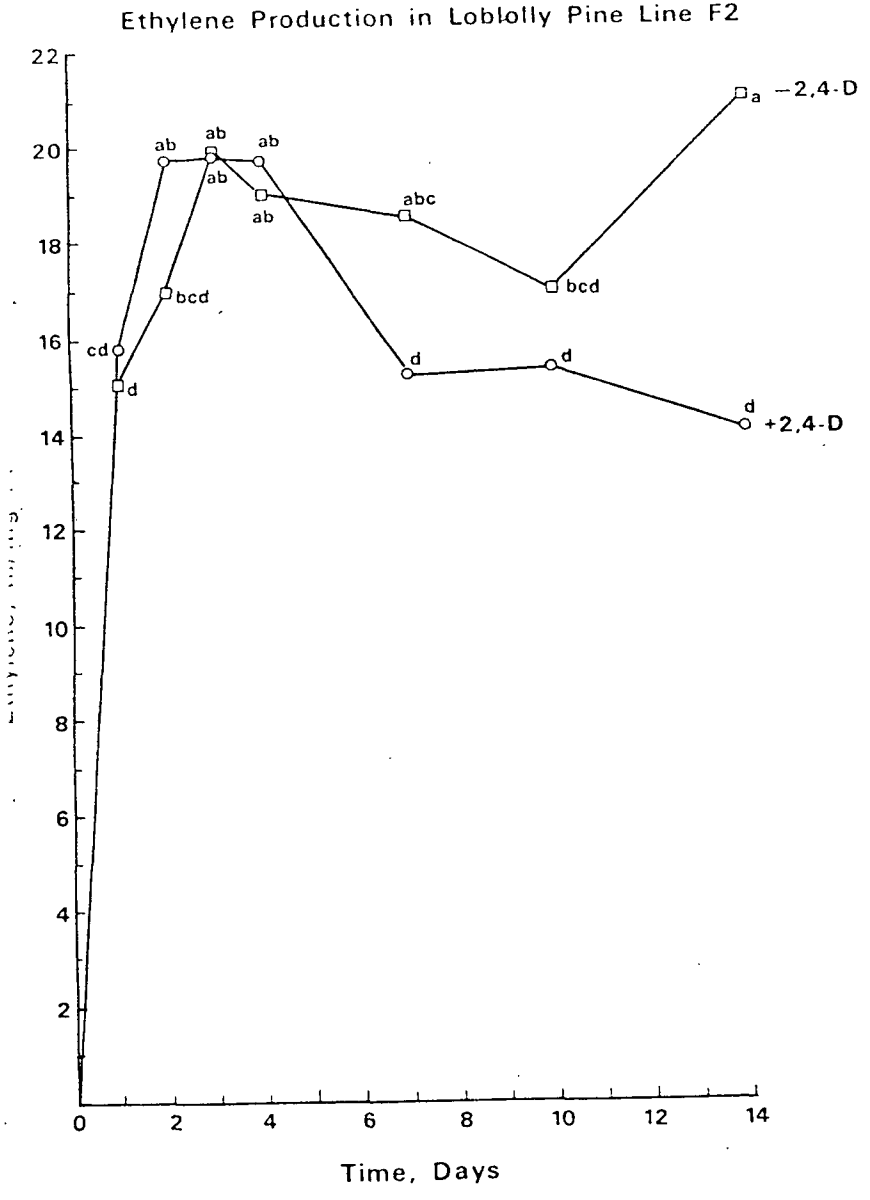
ETHYLENE IN CARROT AND PINE

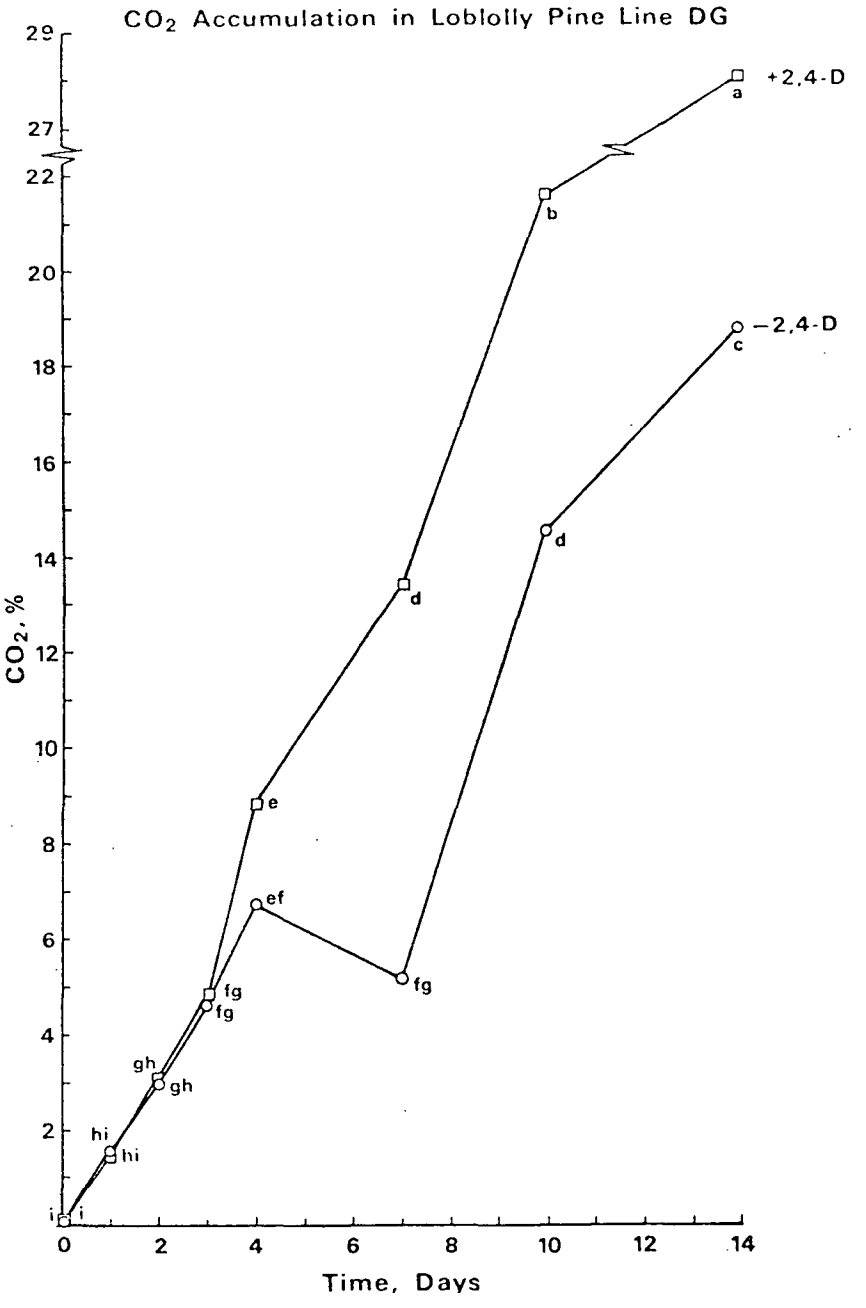
Question: Does the absence or presence of any of the gases produced by pine cell cultures inhibit organized growth?

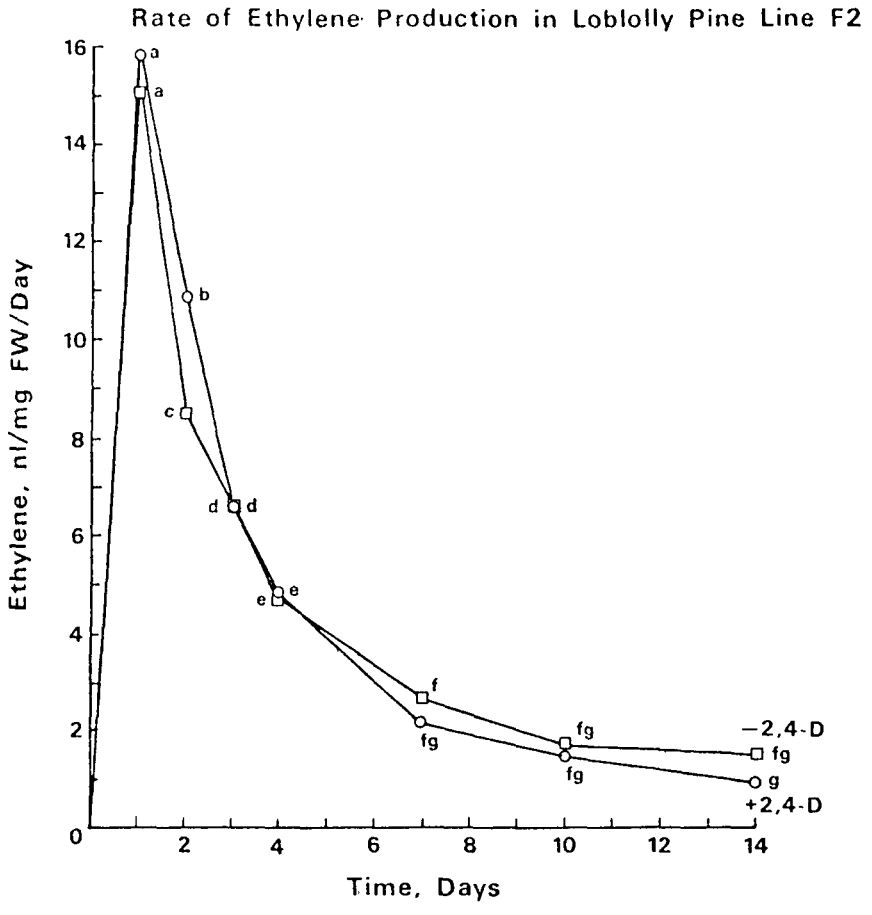
Answer: Excessive early production of ethylene in pine cell suspension cultures may be a factor in inhibiting organized growth. However, CO_2, methanol, and ethanol do not seem to be a problem.


Rate of Ethylene Production in Loblolly Pine Line DG
Ethylene, nl/mg FW
Time, Days
+2,4-D
−2,4-D
a
ab
bc
c
cd
cd
de
e
f
fg
fg
fgh
gh
h


Ethylene Production in Loblolly Pine Line DG
Ethylene, nl/mg FW
Time, Days
+2,4-D
−2,4-D
a
a
b
bc
bc
bc
bc
bcd
cd
bcd
bc
d
d
e
e


Ethylene Production in Loblolly Pine Line F2
22
20
18
16
14
12
10
8
6
4
2
0
2
4
6
8
10
12
14
Time, Days
a −2,4-D
+2,4-D
ab
ab
ab
ab
ab
abc
bcd
bcd
cd
d
d
d
d


CO_2 Accumulation in Loblolly Pine Line DG
29
27
22
20
18
16
14
12
10
8
6
4
2
0
2
4
6
8
10
12
14
CO_2, %
Time, Days
a +2,4-D
b
c −2,4-D
d
d
e
ef
fg
fg
fg
gh
gh
hi
hi
i
i


Rate of Ethylene Production in Loblolly Pine Line F2
Ethylene, nl/mg FW/Day
Time, Days
−2,4-D
+2,4-D

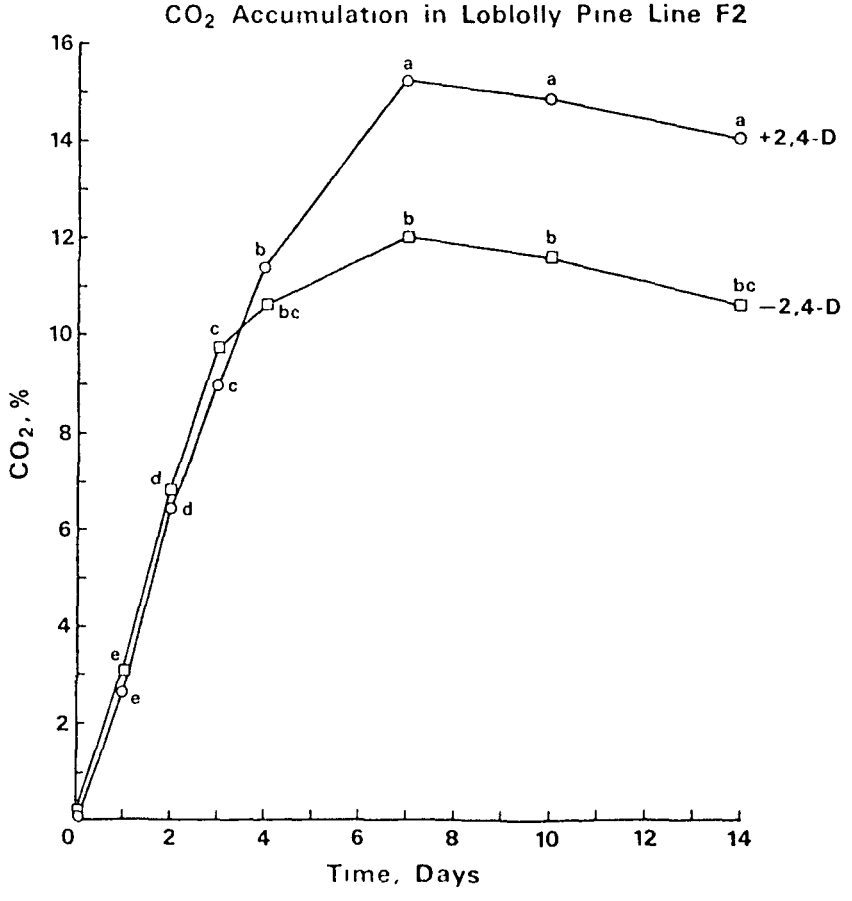

CO2 Accumulation in Loblolly Pine Line F2
CO2, %
Time, Days
+2,4-D
−2,4-D

TABLE 1

Ethylene Production in Wild Carrot

	Ethylene, nl/mg FW	
	+ 2,4-D	- 2,4-D
Day		
0	ND[a]	ND
1	ND	ND
2	ND	ND
3	ND	ND
6	.40	Trace
9	.20	Trace

[a]ND = Not detected.

TABLE 2

Rate of Ethylene Production in Pine and Wild Carrot Cultures

Line	Pine				Carrot	
	DG		F2		245	
	+ 2,4-D	- 2,4-D	+ 2,4-D	- 2,4-D	+ 2,4-D	- 2,4-D
Day	Ethylene, nl/g FW/hr					
1	161	173	659	627		
6					2.78	Trace
7	88.5	73.7	90.7	110.5		

TABLE 3

Ethanol and Methanol Production in Loblolly Pine Line DG

	Ethanol[a]		Methanol[a]	
	+ 2,4-D	- 2,4-D	+ 2,4-D	- 2,4-D
Day				
0	ND[b]	ND	ND	ND
1	ND	ND	ND	ND
2	ND	ND	ND	ND
3	ND	ND	ND	ND
4	ND	ND	ND	ND
7	11.0	5.33	ND	ND
10	42.0	8.33	3.33	ND
14	61.0	27.33	1.11	ND

[a]Parts per million in headgas.

[b]ND = Not detected.

TABLE 4

Ethanol and Methanol Production in Loblolly Pine Line F2

	Ethanol[a]		Methanol[a]	
	+ 2,4-D	- 2,4-D	+ 2,4-D	- 2,4-D
Day				
0	ND[b]	ND	ND	ND
1	ND	ND	ND	ND
2	ND	ND	ND	ND
3	ND	ND	ND	ND
4	ND	ND	ND	ND
7	ND	ND	ND	ND
10	Trace	ND	Trace	ND
14	Trace	ND	Trace	ND

[a]Parts per million in headgas.

[b]ND = Not detected.

TABLE 5

Ethanol and Methanol Production in Wild Carrot

	Ethanol[a]		Methanol[a]	
	+ 2,4-D	- 2,4-D	+ 2,4-D	- 2,4-D
Day				
0	ND[b]	ND	ND	ND
1	ND	ND	ND	ND
2	ND	ND	ND	ND
3	ND	ND	ND	ND
6	18.67	18.67	Trace	3.33

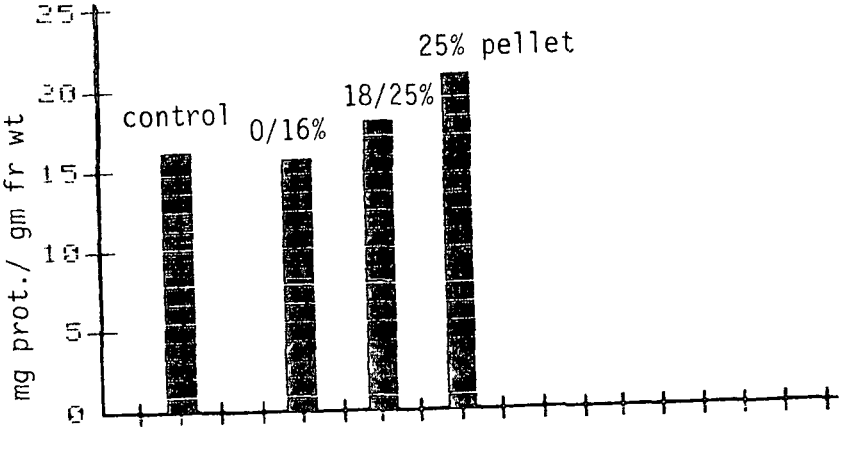
[a]Parts per million in headgas.

[b]ND = Not detected.

PROTEIN CONTENT IN FRACTIONATED LOBLOLLY PINE CELLS

Fraction	mg protein/gm fr wt cells
Control, unfractionated	16.1
0/16% interface	15.6
18/25% interface	18.0
pellet in 25%	20.7

Protein content in fractionated loblolly pine cells[1]



[1]cells separated on Ficoll gradients of indicated concentrations

ENZYMES IN FRACTIONATED SUSPENSION CULTURED LOBLOLLY PINE CELLS[4]

Fraction	Enzyme Activity[1]	
	ADC[2]	ODC[3]
Unfractionated	.014 ± .008	.052 ± .006[a]
0/16% interface	.049 ± .005	.124 ± .016[b]
18/25% interface	.053 ± .037	.088 ± .021[c]
pellet in 25%	.036 ± .023	.097 ± .022[b,c]

[1]nmoles $^{14}CO_2$ per gm fr wt·hr.

[2]No significant differences between ADC means.

[3]ODC values followed by different superscripts are significantly different ($p<.05$).

[4]Cells grown in LM medim containing 2,4-D (2.0 mg/L) and BA (0.1 mg/L) in darkness.

EFFECT OF LIGHT ON ENZYMES IN SUSPENSION CULTURED LOBLOLLY PINE CELLS[c]

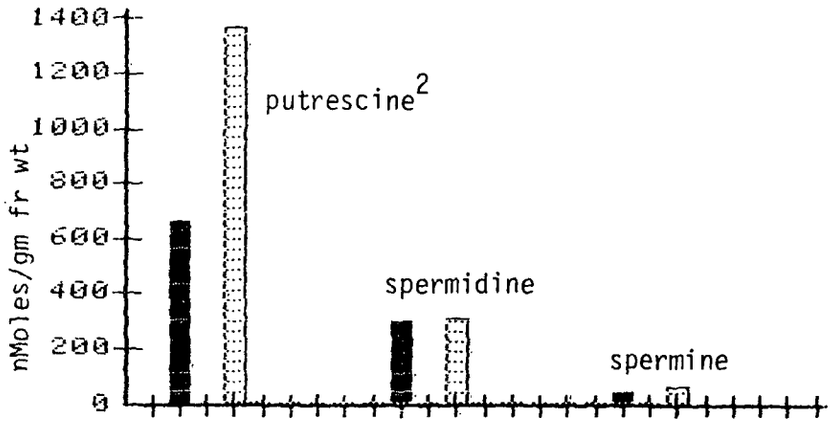
	^{14}C - Arg	Arg + DFMA	Arg + DFMO	^{14}C - Orn	Orn + DFMO
Light	1.70 ± .03[b]	.21 ± .06[a]	1.36 ± .12[a]	.44 ± .02[b]	.16 ± .04[a]
Dark	1.42 ± .11[b]	.09 ± .07[a]	1.13 ± .16[a]	.54 ± .01[b]	.24 ± .01[a]

[a]Significantly different from uninhibited control.

[b]Significant differences between light vs. dark values.

[c]Cells grown for 6 days in LM medium containing 2,4-D (2.0 mg/L) and BA (0.1 mg/L)

Effect of light on polyamines in loblolly pine cells[1]



[1]cells grown for 6 days in LM medium containing 2,4-D (2 mg/l) and BA (0.1 mg/l). Closed bars represent dark, open bars represent light grown cells.

[2]means are significantly different as compared by Duncan's New Multiple Range Test ($p<.05$)

EFFECT OF LIGHT ON POLYAMINES IN LOBLOLLY PINE CELLS[1]

Treatment	Putrescine[2]	Spermidine	Spermine
dark	666	302	45
light	1363	312	56

[1]Cells grown for 6 days in LM medium containing 2,4-D (2 mg/L) and BA (0.1 mg/L). Polyamine levels expressed as nmole/gm fr wt.

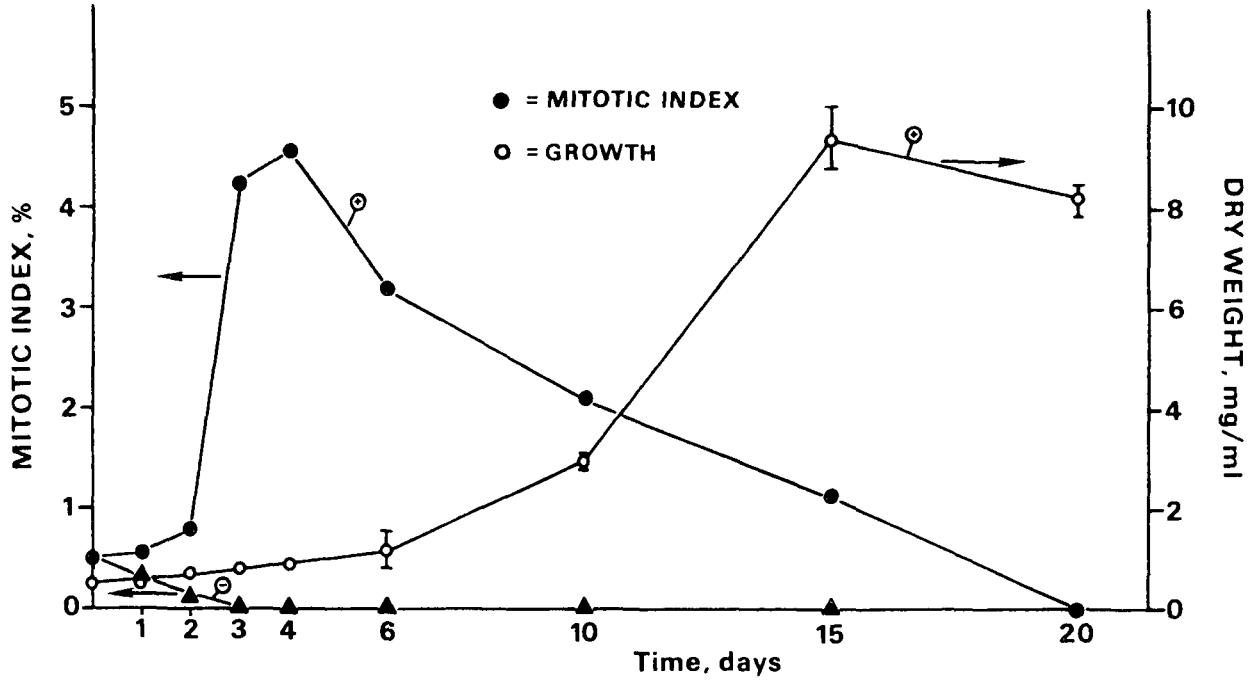
[2]Means (n = 3) are significantly different as compared by Duncan's New Multiple Range Test ($p<.05$).

PINE LAUNCH FACTORS

Q. 1. Are pine suspension cells which fail to grow under launch con ditions dead or merely in a resting or inhibited state?
2. Are there pine cell lines which have the capacity to grow at inoculation densities below 10 µL/mL?

A. 1. It was found that launched quiescent pine cells would resume proliferative growth upon the addition of synthetic auxin provided that the auxin was added during the first week or so of the launch.

2. It has been found that at least two of our pine cell lines have the capacity to grow at an inoculation density of 5 µL/mL (carrot is normally launched at at 0.5 µL/mL).



The mitotic indexes and associated growth curve for a loblolly pine cell suspension inoculated at 10 µL/mL and growing in LM ± 2,4-D. Only the + 2,4-D growth curve is shown; the cells did not grow without 2,4-D.

Growth recovery of pine suspension cells after exposure to auxin-free medium.

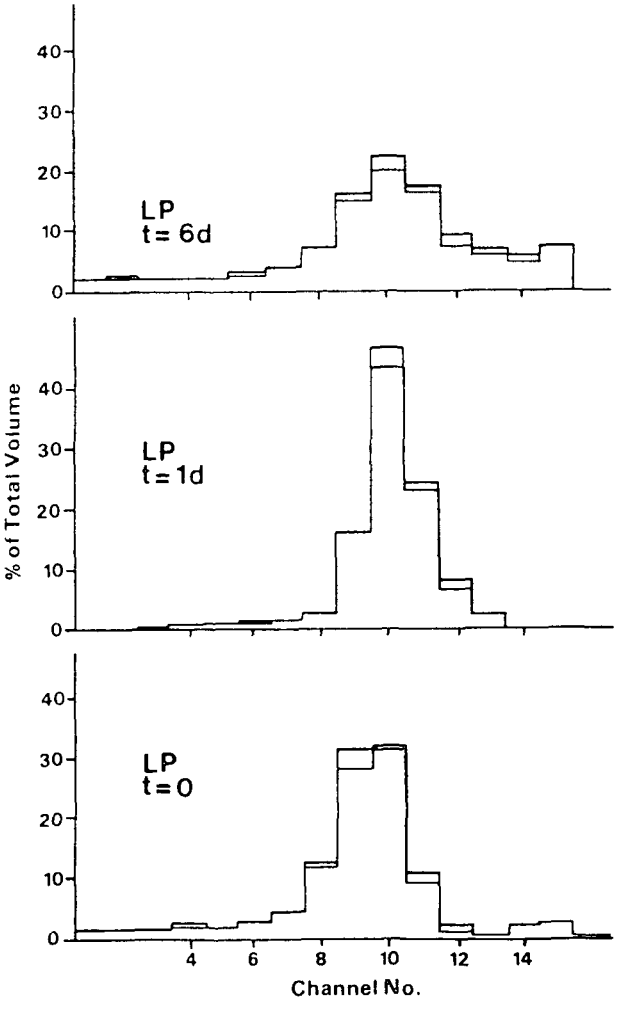
Time of 2,4-D Spike, days	Growth after Spike: Mean Dry Wt., mg/mL at day 21[a]
0 (control)	1.7^{f}
1	4.3^{de}
2	6.0^{c}
3	7.5^{ab}
4	7.9^{a}
7	5.3^{cd}
9	1.4^{fg}
11	0.5^{fg}
15	0.5^{fg}

[a]ANOVA and Duncan's Multiple Range Test for mean dry wt. differences due to spike time were run; values with a common superscript are not significantly different at the 95% confidence level.

Effect of inoculation density on growth of pine cell suspensions.

Cell Line ([2,4-D], mg/L in stock culture)	Inoculation Density, µL/mL	Growth[a] Mean Dry Wt., mg/mL ± SD at day 14
F-2 (2.0)	5	0.9 ± 0.2
	10	3.9 ± 0.8
	15	8.6 ± 1.2
	30 (parental)	10.8 ± 0.9
34-10 (0.5)	5	1.2 ± 0.3
	10	5.5 ± 1.1
	15	11.1 ± 3.8
	30 (parental)	13.4 ± 1.2
34-10 (2.0)	5	0.8 ± 0.1
	10	3.3 ± 1.0
	15	14.1 ± 0.6
	30 (parental)	13.3 ± 1.1

[a]Inoculum mean dry wt. determined for line F-2 was 0.029 mg/mL (n = 4). ANOVA and Duncan's Multiple Range Test (95% confidence level) indicated significant growth of all samples, including those at 5 µL i.d., relative to starting inoculum wt.



UNMONITORED LAUNCHES

Q. Have any of the new cell lines initiated from excised immature loblolly pine embryos in 1983 and 1984 exhibited a capacity for morphogenesis?

A. Although some of the 1983 cell lines are still extant, no definite morphogenic potential has been noted. The same must be stated for the 1984 material although evaluation is not yet completed in this case.

Launch matrix for 1983 cell lines.[a]

Basal Medium	Growth Regulators, mg/L		
	None	0.01 NAA/0.1 BAP	0.01 NAA/1.0 BAP
LM $\bar{c}$ 2/3 sucrose, 1/2 NH_4NO_3 + 1 mM agmatine	x	x	x
1/2 LM + 1 mM agmatine	x	x	x

[a]Cell lines launched were: 34-10, 239D-F (pooled source), 34-3G, 218-F-2 (pooled source), 35-7F, 35-3F, 239D-G (pooled source), 31-1-F, 38-5G, 31-6F, 23-1G, 218-8-2G (pooled source). Proliferative growth controls were also run (see text).

Agar launch protocols for 1984 initiations of loblolly pine.

No.	Basal Medium	Growth Regulators[a]	Additives	Other
1	1/2 LM	None	gln (400 mg/L)	--
2	1/2 LM	1.0 BAP/0.1 NAA	gln (400 mg/L)	--
3	1/2 LM	1.0 BAP/0.1 NAA	gln (400 mg/L)	Far red light
4	1/2 LM	None	gln (400 mg/L)	--
			0.25 mM spermidine	--
			LP seed extract	--
5	RM-2[b]	None	None	--
6	RM-2[b]	0.1 BAP	None	--
7	RM-2[b]	None	LP seed extract	--
8	RM-2[b]	None	5mM arg + 1 μM met	--
9	Spruce[c]	None	gln (500 mg/L)	--
10	Spruce[c]	0.5 BAP/0.05NAA	gln (500 mg/L)	--
11	Walnut[d]	1.0 BAP/2.0 kinetin	gln (250 mg/L)	--
		0.1 IBA		
12	Coffee[e]	0.5 Kinetin/0.05 NAA	None	--

[a] mg/L.

[b] RM-2 medium consists of (in mg/L): KH_2PO_4 (170), KCl (745), $CaCl_2 \cdot 2H_2O$ (166), KI (4.15), H_3BO_3 (31), $MnSO_4 \cdot H_2O$ (21), $ZnSO_4 \cdot 7H_2O$ (8.6), $Na\ MoO_4 \cdot 2H_2O$ (1.25), $CuSO_4 \cdot 5H_2O$ (0.5), $CoCl_2 \cdot 6H_2O$ (0.5), $FeSO_4 \cdot 7H_2O$ (27.8), $Na_2EDTA \cdot 2H_2O$ (37.3), myo-inositol (100), nicotinic acid (0.5), pyridoxine·HCl (0.1), thiamine·HCl (0.1), gln (1460), GSH (31), folic acid (0.5), biotin (0.5), $MgCl_2 \cdot 6H_2O$ (203), and sucrose (20,000).

[c] Simola, L. K., and Honkanen, J., Physiol. Plant 59:551-61(1983).

[d] Driver, J. A., and Kunizuki, A. H., Hortsci. 19:507-9(1984); except K_2SO_4 not $KHSO_4$ and $ZnNO_3$ changed to $ZnSO_4$.

[e] Sondahl, M. R., and Sharp, W. R., Z. Pflanzenphysiol. 81:395-408(1977).

Liquid launch protocols for 1984 initiations of loblolly pine.

No.	Basal Medium	Growth Regulators[a]	Additives
1	1/2 LM	None	gln (500 mg/L)
2	1/2 MS	None	gln (500 mg/L)
3	1/2 MS	1.0 BAP/0.01 NAA	gln (500 mg/L)
4	1/2 MS	1.0 BAP	gln (500 mg/L)
		2.0 Kinetin	
		0.1 IBA	
5	1/2 MS	1.0 BAP	gln (500 mg/L)
		2.0 Kinetin	

[a] mg/L.

MODEL SYSTEMS AND/OR MODEL SPECIES RESEARCH

Model systems research involves biochemical investigations that we feel will help us:

- better understand the biochemistry of somatic embryogenesis
- develop biochemical markers for monitoring conifer somatic embryogenesis

Feirer

A. Polyamine Research

Hypothesis: Polyamines are important in plant development and could be one of the factors limiting somatic embryogenesis in conifers.

Objective:
1. Develop methods of monitoring polyamines.
2. Establish the importance of polyamines in plant development.
3. Determine which of the polyamines seems to be most important in conifers.
4. Develop methods for manipulating polyamines in conifer cell lines.

Status:

1. Appropriate methods have been developed.
2. Inhibitor studies have demonstrated the importance of polyamines, particularly spermidine, in plant development.
3. The role of light on enzyme activity and polyamine biosynthesis has been studied.
4. Investigations are under way on manipulating polyamines in wild carrot and conifer cell lines.

Research Priorities

Next Six Months

1. Make additional comparisons of polyamine metabolism in intact plants and *in vitro*.
2. Examine polyamine levels during loblolly pine organogenesis.
3. Determine role of polyamines during *in vivo* embryo development.
4. Acquire additional information on the relationship between polyamines and ethylene metabolism.
5. Evaluate further the effect of light on enzyme activity and polyamine biosynthesis.

Research Priorities

Next Six Months

6. Compare polyamines, proteins, enzymes, etc. in additional subpopulations of cells.

7. Evaluate the effect of polyamines on protein synthesis.

Future Plans

1. Isolate/select cell lines exhibiting altered polyamine metabolism.

2. Use radio-labelled DFMA, DFMO to quantitate and characterize ADC/ODC.

3. Evaluate the effect of growth regulators on polyamines and protein synthesis.

Johnson & Noland

B. Phenolics of Cell Culture

Hypothesis: Cell cultures may be inhibited from forming organized structure by the build-up of phenolics. Additionally, certain phenolics may promote formation of organized structures when present at proper levels and ratios.

Objective:
1. Develop a procedure for separation and quantification of phenolics.
2. Determine base line levels of phenolics in loblolly pine and wild carrot cultures.
3. Examine the influence on phenolics on embryogenesis.

Status:
1. A HPLC procedure for separation and quantification of several phenolics has been achieved.
2. Pine cells were found to have more persistant and higher levels of PAL enzyme activity than wild carrot cells. PAL is a key enzyme associated with high levels of tannins.
3. Low levels of several phenolic compounds added to the media did not reduce growth of loblolly pine and wild carrot nor embryogenesis in wild carrot. Results with ferulic acid indicate oxidation is probably involved when toxicity is observed.

Research Priorities

Next Six Months

1. Determine base line levels of phenolics in loblolly pine and wild carrot cultures.

2. Extract and quantify phenolics in loblolly pine cell suspension lines growing in the light, dark and with high and low sucrose.

3. Conduct PAL inhibitor studies (AOPP and AOAA) as a way of manipulating phenolic levels in loblolly pine and wild carrot.

Future Plans

1. Run additional radio tracer experiments on phenolic metabolism in wild carrot and loblolly pine cell cultures.

2. Look into the potential of using ferulic acid and similar phenolics to improve cell line quality.

Noland

C. Gas Sampling Cell Cultures for CO_2, Ethylene, Ethanol and Methanol.

Hypothesis: Ethylene may be necessary (especially during initiation) to produce cells which are competent. The other gases, CO_2, ethanol, and methanol may become inhibitory if they build up to sufficient levels.

Objective:
1. Develop appropriate measurement techniques for ethylene and other gases.
2. Establish base line levels of gases for wild carrot and loblolly pine cell lines.
3. Evaluate the importance of ethylene and other gases in somatic embryogenesis.

Status:
1. Measurement techniques have been worked out.
2. Preliminary measurements were made on wild carrot and loblolly pine cultures.
3. Under standard growth conditions, CO_2 build-up was greater in loblolly pine than cultures wild carrot.
4. When loblolly pine culture tubes were sealed significant levels of ethylene, ethanol, methanol and CO_2 accumulated by day 15.

Research Priorities

Next Six Months

1. Additional "gas build-up" experiments are planned with loblolly pine and wild carrot using gas impermeable closures.

2. Studies involving ethylene removal and additions to cell cultures are planned.

3. Research on effects of growth regulators on ethylene production in loblolly pine will be initiated.

Johnson

D. Natural Extracts.

Hypothesis: Unrecognized growth and development factors in natural pine embryos may be extractable and used to promote growth and development of cultured pine cells.

Objective: Prepare extracts and test their effects on growth and development of wild carrot (screen for toxicity) and pine cell suspensions.

Status:

1. Initial tests yielded one high molecular weight fraction which consistently stimulated wild carrot embryogenesis.

2. This same fraction produced mixed results in both growth and biological markers for pine cells.

Research Priorities

1. Subfractions of both the high and low molecular weight material will be evaluated.

Johnson

E. Tracer Studies of Metabolic Problems

Hypothesis: Radioactive tracers will allow pinpointing metabolic problem areas in cultured pine cells, relative to model system cells.

Objective: Use key radiolabeled metabolic precursors to compare the fate of these precursors in pine cells and model cells as a function of growth and development.

Status: 1. Recent experiments of this type have involved:

- polyamine metabolism
- two studies probing the distribution of phenylalanine into proteins vs. phenolics in pine cells

Research Priorities

1. Investigate the distribution of phenylalanine into proteins vs. phenolics in wild carrot.

2. Study the metabolic fate of several labeled carbon sources in pine vs. carrot, with emphasis on the pentose phosphate cycle and nucleic acid biosynthesis.

Johnson

F. Growth Regulator Research (Endogenous).

Hypothesis: Most GR research involves adding exogenous growth regulators to cells in which endogenous levels are unknown. It is postulated that more intelligent manipulation of pine somatic embryogenesis would result if analytical data on endogenous levels were available.

Objective: Develop the methodology and use it to determine and compare endogenous growth regulator (several types) levels in model cells and pine cells as a function of growth and development.

Status: 1. Personnel limitations have resulted in minimal activity in this research area. Student research on the IAA/indole pathway has opened the possibility of fruitful research in this metabolic area.

Research Priorities

Next Six Months

1. Develop pine cell lines using only naturally occurring growth regulators.

Future Plans

1. Develop an appropriate "in-house" method of determining endogenous levels of IAA and related metabolic products.

2. Initiate base line evaluations of endogenous levels of IAA and other natural growth regulators in loblolly pine, coffee and wild carrot lines and developing embryos.

3. Use radiotracers to study the synthesis and fate of natural growth regulators.

Johnson

G. Growth Regulator Research (Synthetic)

Hypothesis: The synthetic GR 2,4-D was developed for use with herbaceous plants, not conifers. There may be other synthetic GR's more suitable for use with conifers.

Objective: 1. Evaluate alternative synthetic growth regulators to replace 2,4-D in loblolly pine and Douglas fir cell cultures.

Status:

1. Fifteen growth regulators were evaluated for use as alternatives to 2,4-D and a few were promising enough to warrant further testing.

2. Related student research resulted in evidence that the accumulation of 2,4-D by cell lines, as the result of repeated subculturing, may result in cell line incompetence.

Research Priorities

Next Six Months

1. Attempt to generate loblolly pine cell lines that will grow without 2,4-D.

2. Generate and maintain loblolly cell lines on three or four of the most promising alternative growth regulators.

3. Look into the influence of synthetic growth regulators on ethylene production.

Johnson

H. Energy Research.

Hypothesis: Pine cells in suspension may fail to generate and maintain sufficient energy levels for biosynthetic needs.

Objective: Determine levels of ATP and energy charge in model systems and pine cell suspensions and compare for signs of energy deficiency.

Status:

1. Data compared on a fresh weight basis does not suggest any energy problem in pine cultured cells.

2. It appears pine cells are not using their energy reserves when subjected to enbryogenesis protocals.

Research Priorities

1. This research is on hold until we can express the data on a per cell basis, to see if this affects interpretation.

Johnson

I. Redox Research.

Hypothesis: Pine cells in suspension may not regulate their internal redox status in the same manner as model systems capable of embryogenesis.

Objective: Determine the levels of ascorbic and dehydroascorbic acids and compare for signs that the pine cells differ from model cells in the manner in which internal redox state fluctuates during growth and development.

Status:

1. Measurements of ascorbic acid, dehydroascorbic acid and glutathione were made on wild carrot to obtain an estimate the redox status of this system.

2. Similar measurements were made during natural pine embryogenesis and for pine cell suspension during attempts at producing embryos.

3. Data available at this time suggests that the pine cells are too oxidizing during a time period critical for embryo development.

Research Priorities

1. Wild carrot research on redox status will be completed under thesis research.

2. Existing data will be recalculated on a per cell basis where possible.

3. Further measurement will be made on pine cell lines using a redox electrode and an oxygen electrode.

4. Attempts will be made to correct the apparent problem.

J. Cell Growth Studies.

Hypothesis: Cell number, cell size and cell division rates are related to biochemical events in a more significant way than is the mass measurements of wet and oven dry weight.

Objective:
1. Develop a procedure to disperse cell clumps into a suspension of individual cells without seriously impairing the integrity of the cells.
2. Measure cell number per unit volume, cell size and the rate of cell division in dispersed cell suspensions throughout the course of an experiment.
3. Relate biochemical events during the experiment to these cell parameters, as well as the conventional wet and oven dry weight measurements.

Status:
1. Cell counting technique has been perfected.
2. A mitotic index measurement technique has been perfected.
3. Preliminary counting and mitotic index information has been obtained on loblolly pine.

Research Priorities

Next Six Months

1. Evaluate both approaches using carrot and loblolly pine cell lines of varying quality.
2. Investigate the reasons for cell clump dispersal difficulties encountered in launched pine cells.

Future Plans

1. Use methods in future work when appropriate, to evaluate cell line treatments and stages of somatic embryogenesis.

Verhagen, Johnson
& Wann

Objective I - Initiation Research

Hypothesis: Embryogenetic cell lines are required as a starting point somatic embryogenesis.

Objective: Generate and maintain embryogenetic cell lines.

Status:

1. Research emphasis has been on immature tissue sources and growth regulators.
2. Several hundred new cell lines were generated from immature loblolly pine embryos.
3. Additions of polyamines and natural conifer extracts failed to improve cell line quality.
4. Several synthetic auxins were tested that produced comparable growth response and may be less disruptive to subsequent development than 2,4-D.
5. Cell lines were initiated using "non-2,4-D" growth regulators.
6. New cell lines have been produced that grow at low 2,4-D levels and low inoculation densities.

Research Priorities

Next Six Months

1. Generate new cell lines from immature embryos and first year cones.

2. Generate new cell lines from protoplasts and cell lines using proven organogenic seed sources.

3. Develop techniques for readily separating cell lines by clump size, and for growing cell lines at low inoculation density.

4. Initiate and grow additional cell lines without 2,4-D.

Future Plans

1. Generate haploid cell lines (from pollen, endosperm).

2. Select cell lines for high (or low) production of chemicals important to embryogenesis.

3. Develop photoautotropic cell lines.

Johnson, Wann, Feirer
& Verhagen

Objective II - Embryogenesis Research

Hypothesis: Given embryogenetic cell lines, - somatic embryogenesis can be accomplished by manipulation of critical environmental factors.

Objective: Develop controlled reproducible procedure for obtaining somatic embryogenesis in conifers.

Status: 1. Earlier work included:

- BA pulse treatment
- Launching newly initiated lines
- Natural extracts
- Polyamine manipulation

Status: 2. Recent investigations include:

- N-benzyladenine uptake study
- Unmonitored launch of 1983 cell lines
  -- 12 best LP cell lines and six media
- Unmonitored launch of 1984 cell lines
  -- 2000 LP explants on 12 media
- Launches where growth regulator removal and inoculation density were investigated

Research Priorities

Next Six Months

1. Establishing monitored launches aimed at the correction of apparent deficiencies.

2. Conducting unmonitored launch experiments incorporating a combination of promising factors.

3. Starting launches in which cell clump size is regulated and inoculation densities are lower.

Future Plans

1. Initiating launches of light grown lines, lines with new growth regulators and lines with low phenolic levels.

2. Conducting launches with conifers other than loblolly pine, e.g., Douglas-fir, and Pinus oocarpa, as available.

PLANNED PROJECT STAFF ADDITIONS

1. Section Leader - Tissue Culture Background
2. Tissue Culturist - Experienced Researcher
3. Tissue Culturist - Newly Trained
4. Biochemist

TISSUE CULTURE MANPOWER COMMITMENT - FY 84/85

Research Area	1st 6 Month, Man Years Ph.D.	Tech. Asst.	2nd 6 Month, Man Years Ph.D.	Tech. Asst.
Model Systems	1.1 (61%)	1.5 (27%)	1.3 (65%)	1.9 (32%)
Objective I	0.4 (22%)	1.5 (27%)	0.4 (20%)	1.6 (27%)
Objective II	0.2 (11%)	0.9 (16%)	0.2 (10%)	1.0 (17%)
Objective III	0 --	0 --	0 --	0 --
Objective IV	0.1 (6%)	0.2 (4%)	0.1 (5%)	0.1 (2%)
Maintenance	--	1.4 (25%)	--	1.3 (22%)
Totals	1.8 (100%)	5.5 (100%)	2.0 (100%)	5.9 (100%)

RELATED RESEARCH

COOPERATIVE INVESTIGATIONS

1) North Carolina State - a cooperative study with Dr. Ralph Mott and Dr. Henry Amerson on variation of polyamine levels during organogenesis of loblolly pine.

2) University of New Hampshire - A cooperative study with Dr. Subhash Minocha on the use of nuclear proteins (histones) as biochemical markers for monitoring somatic embryogenesis.

3) St. Norbert College - A cooperative study with Dr. John Phythyon on the role of methionine and S-adenosylmethionine on potential embryogenesis of loblolly pine cell suspensions.

RELATED RESEARCH

STUDENT RESEARCH

1) Steven Wann - A tissue culture oriented Ph.D. program entitled "Selection of Mammatoxin-Resistant Aspen via Plant Tissue Culture."

2) Brent Earnshaw - A biochemically oriented Ph.D. program entitled "An Investigation into the Functions of glutathione and Ascorbic Acid in Growth and Development of Wild Carrot Suspension Cultures and Plants."

3) Peter Ryan - An independent study (MS) topic entitled "An Investigation of Methyleneoxindole (MEOI) and its Metabolism in Conifers."

RELATED RESEARCH

STUDENT RESEARCH (Continued)

4) Luke Nealey - An organic chemistry oriented Ph.D. program entitled "Isolation and Characterization of Xyloglucan from Suspension Cultured Loblolly Pine Cell Medium."

5) Rene Kapik - An independent study (M.S.) topic entitled "Phenolic Components of the Primary Cell Wall and Their Possible Role in the Regulation of Growth."

6) Russell Feirer - A biochemically oriented Ph.D. program investigating the role of polyamines and associated enzymes in plant development (in cooperation with the University of Wisconsin, Madison).

PROJECT TITLE: The Mass Production of Conifers -- Loblolly Pine and Douglas-fir

PROJECT STAFF: D. Einspahr, M. Johnson

PRIMARY AREA OF INDUSTRY NEED: Raw Materials

PROGRAM AREA: Increased wood production by embryogenesis and bioengineering

Date:	2-7-85
Budget:	$570,000*
Period Ends:	6-30-86
Project No.:	3223
Approved by VP-R:	

PROGRAM GOAL: To increase significantly the productivity of our forests by the propagation of superior trees.

PROJECT OBJECTIVE/GOAL:

The overall objective is the mass production of conifers. The near-term objective is the development of a procedure for producing plantlets from single cells or small groups of cells.

PROJECT RATIONALE:

Major increases can be obtained in tree growth and forest production through the clonal propagation of "elite" trees and through the creation of new genetic combinations. Planned genetic combinations are ones that are difficult to produce using conventional techniques but are anticipated to result in individuals of exceptional disease resistance and special site and/or climatic adaptability. Production of plantlets from cell suspensions will open the way to the badly needed genetic gains described above through genetic engineering. The cell suspension approach forms the basis for a second-generation technology that is considered to some day replace the existing practice.

RESULTS TO DATE:

Appropriate new media have been developed for initiating callus production and for growing cells in suspension. Procedures for establishing appropriate cell lines have been developed. Biochemical and morphological characterization of embryogenesis is under way. Use of wild carrot somatic embryogenesis and natural Douglas-fir and loblolly pine embryo development as model systems, have assisted in establishing media change requirements and in developing needed biochemical markers. Loblolly pine organogenesis and coffee somatic embryogenesis procedures have also been worked out and these will also be used as part of our research in the development of biochemical markers. Excised conifer embryo investigations have been used to determine the nutrient requirements of developing embryos. Inhibitor studies have demonstrated the importance of several polyamines in embryogenesis and determined that polyamine synthesis in wild carrot is controlled mainly through the arginine-agmatine pathway. Recent polyamine inhibitor investigations seem to indicate spermidine may be the polyamine most involved in plant development. Alternative procedures for modifying free amino acid and polyamine levels have been determined. Studies of natural pine embryogenesis indicate polyamines play an essential role in the development of pine embryos in maturing cones. Tests of the new LM medium for growing conifer cells in suspension demonstrated the medium can also be used to produce somatic embryogenesis in wild carrot. New immature embryo cell lines

*Excludes additional funding from member companies.

have been produced and they are being monitored to determine if they have an improved potential for embryogenesis. The immature seed extracts that improved wild carrot embryogenesis are being evaluated on loblolly pine cell lines. Research into energy charge levels of the wild carrot system and loblolly pine cell lines revealed the pine cells in culture have a high energy charge and should have no problem with adequate ATP levels to drive biosynthesis. Ascorbic acid investigations indicate that wild carrot cultured cells may have more control of their internal redox status than do pine cells, particularly the old cell lines. Investigations on screening synthetic auxins for an alternative for 2,4-D in pine cell cultures have turned up two compounds that have promise. A comprehensive, updated long-range research plan has been developed that includes ways of expanding the program and accelerating progress. The first steps aimed at implementing the new plan have been taken.

PLANNED ACTIVITY FOR THE PERIOD:

Plans for the coming year include additional model systems research on (1) comparing polyamines metabolism in loblolly pine, coffee and wild and domestic carrot; 2) determining the roles of polyamines in loblolly pine organogenesis and in in vivo embryo development of red pine, and wild and domestic carrot; 3) evaluating the relationship between polyamines and ethylene biosynthesis; 4) determining the effects of growth regulators on ethylene production in loblolly and wild carrot lines, and levels of ethylene during loblolly pine and/or Douglas-fir organogenesis; 5) examing the role of phenolics in loblolly pine cell growth and quantifing the effects of light vs. dark culture conditions and of sucrose levels on phenolic production in loblolly pine callus; 6) localizing metabolic problem areas using tracer techniques; 7) evaluating further the importance of redox in cell cultures using redox electrodes, oxygen electrodes and other techniques; 8) examining further the usefulness and chemical characteristics of high and low molecular weight extract fractions from immature loblolly pine seeds. Research on generating and maintaining competent cell lines is expected to include (1) generation of new cell lines from immature embryos, protoplasts, haploid tissue, "bumpies" and proven seed sources; (2) initiation and growth of cell lines with auxins other than 2,4-D; (3) selection and growth of cell lines capable of the production of high levels of specific chemicals; and (4) production of photoautotropic cell lines. Conifer somatic embryogenesis studies are expected to include (1) establishing monitored launches aimed at the correction of apparent deficiencies, (2) conducting unmonitored launch experiments incorporating a combination of promising factors, (3) starting launches in which cell clump size is regulated and inoculation densities are lower, (4) initiating launches of lines grown in the light, lines with new growth regulators and lines with low phenolic levels, and (5) conducting launches which employ conifers other than loblolly pine, e.g., Douglas-fir, and Pinus oocarpa, as available.

POTENTIAL FUTURE ACTIVITIES:

Future emphasis, when somatic embryogenesis is a reality, will be on plantlet transfer to soil, developing procedures for determing the fidelity of somatic embryos and research related to genetic engineering (i.e., production of haploid cell suspension, protoplast fusion, etc.).

SHORT TERM GOALS:

Goals for 1985/86 in the conifer tissue culture program:

1. Complete the biochemical characterization (free amino acids, polyamines, and proanthocyanidin) of:

   (a) Wild carrot somatic embryogenesis model system, and
   (b) The conifer natural embryogenesis model system.

2. Determine factors critical to the conversion of arginine to polyamines.

3. Work out methods of controlling polyamine and proanthocyanidin levels in rapidly growing cell lines.

4. Generate and evaluate several new, very juvenile cell line sources including immature embryos, haploid tissue, and protoplasts for their embryogenetic capability.

5. Generate loblolly pine cell lines that can be grown with alternate growth regulators (NOAA, IAA, IBA, etc.)

6. Evaluate and select appropriate methods for determining growth regulator levels (IAA, IBA, GA, NOAA, BAP, etc.) in tissue culture samples.

7. Establish patterns of change in growth regulator levels present during natural conifer and wild carrot somatic embryogenesis.

Growth regulator levels appear to be critical during the early stages of embryogenesis. Short term goals 5, 6 and 7 provide additional details on the first phases of a planned growth regulator research program. Additional manpower will be required to complete goals 6 and 7.

STUDENT RESEARCH

1. Steven Wann - A tissue culture oriented Ph.D. program entitled "Selection of Mammatoxin-Resistant Aspen via Plant Tissue Culture."

2. Brent Earnshaw - A biochemically oriented Ph.D. program entitled "An Investigation into the Functions of Glutathione and Ascorbic Acid in Growth and Development of Wild Carrot Suspension Cultures and Plants."

3. Peter Ryan - An independent study (MS) topic entitled "An Investigation of Methyleneoxindole (MEOI) and Its Metabolism In Conifers."

4. Luke Nealey - An organic chemistry oriented Ph.D. program entitled "Isolation and Characterization of Xyloglucan from Suspension Cultured Loblolly Pine Cell Medium."

5. Russell Feirer - A biochemically oriented Ph.D. program investigating the role of polyamines an associated enzymes in plant development (in cooperation with the Univ. of Wisconsin, Madison).

PROJECT TITLE: Development of Analytical Techniques for Characterizing Wood & Fiber and Related Exploratory Research

PROJECT STAFF: Section 30

PRIMARY AREA OF INDUSTRY NEED: Raw Materials

PROGRAM AREA: Special Competence

Date: 2/7/85
Budget: $80,000
Period Ends: 6-30-86
Project No.: 3501
Approved by VP-R:

PROGRAM GOAL: Increase the supply and improve the utilization of the fibrous raw materials.

PROJECT OBJECTIVE:

Evaluate and/or develop the analytical techniques required to meet Institute and member company demands for characterizing wood and fiber. Investigate novel ideas and develop supporting information to justify dues-funded projects having the objective of expanding the industry's source of useable fibers.

PROJECT RATIONALE:

Improved utilization of the available supply and investigations into new sources of fiber requires having adequate analytical techniques for characterizing the original untreated source of fiber and the influence that chemical and mechanical action has on the usefulness of these new sources of fiber. These same techniques are expected to be useful in evaluating present and new pulping, bleaching, and refining procedures. Additionally, researchers are encouraged to devote part of their time to developing novel ideas that will result in new analytical techniques and/or new research projects. These exploratory studies are expected to be in the wood and fiber science, tree physiology, biochemistry, and forest genetics area.

RESULTS TO DATE:

Progress in the wood and fiber science area relates primarily to improving our capabilities in characterizing cellulose fibers at the molecular level. The electron microscopy laboratory has two state-of-the-art electron microscopes: a scanning electron microscope (SEM) and a scanning transmission electron microscope (STEM). Besides offering high resolutions imaging capabilities, both scopes can provide qualitative and quantitative elemental analyses. The SEM can detect and measure all elements having an atomic number equal to or greater than that of boron (5) by using an energy dispersive spectrometer (EDS) or a wavelength dispersive spectrometer (WDS). The STEM can determine all elements having an atomic number greater than that of sodium (11) by using an EDS. Work during the past year brought all the equipment on line and established all basic procedures.

Isozyme procedures employing the perioxidase enzyme system demonstrated the potential of determining the "relatedness" of parent trees and suggested the need for additional research to make the approach as definitive as required. Of 25 enzyme systems evaluated, two now appear very promising; peroxidase and acid phosphatase. Additionally, banding patterns of total proteins show promise for use in determining relatedness. A biological similarity index system has been

modified to compare enzyme banding patterns statistically and provide an estimate of relatedness of parent trees. The most recent use of this approach has been to use banding patterns to check the genetic fidelity of plantlets produced via somatic embryogenesis. Comparisons are underway which include comparing isozyme patterns of cell suspensions, callus, embryos and tissue culture generated wild carrot plantlets.

Mycorrhizae investigations have resulted in the development of a suitable inoculation procedure and the establishment of two organisms (Pisolithus tinctorius and Suillus grevillei) on larch that appear worthy of field testing. Additional field collections of mycorrhizae forming organisms were made in 1984 and they are being cultured for future use.

PLANNED ACTIVITIES FOR THE PERIOD:

A. For the wood and fiber science area, preliminary molecular level characterization of fiber surfaces that have been treated in several ways (pulping and refining) will be evaluated.

B. Isozyme procedures for determinng relatedness of parent trees will be discontinued until additional trees of known origin and relatedness become available. The work on using isozymes and protein banding patterns to evaluate tissue culture plantlet fidelity will be continued, time permitting.

C. Parent trees and full sib progeny will be screened for toxin resistant individuals using the procedures described in Steven Wann's Ph.D. thesis entitled "Selection of mammatoxin-resistant aspen via plant tissue culture."

POTENTIAL FUTURE ACTIVITIES:

A. Evaluate the potential of:

    1. High resolution imaging of fiber surfaces which have undergone mechanical or chemical degredation.

    2. Quantification and Z-direction distribution of inorganic fillers and coating materials in paper, and

    3. Determination of lignin distributions in fibers using the bromination reaction.

B. Examine the usefulness of the isozyme approach to "relatedness" using full-sib progeny and additional half-sib populations.

C. Establish a field test with European and/or hybrid larch that would evaluate the growth increases associated with the presence of mycorrhizae.